DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

68/2018

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Alterations and Impact of Thrombin Generation and Clot Formation in Solvent/Detergent Plasma, FXIII Deficiency and Lysinuric Protein Intolerance

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UNIVERSITY OF HELSINKI AND
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Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis 68/2018

Doctoral Program in Clinical Research, Faculty of Medicine
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ALTERATIONS AND IMPACT OF THROMBIN GENERATION AND CLOT FORMATION IN SOLVENT/DETERGENT PLASMA, FXIII DEFICIENCY AND LYSINURIC PROTEIN INTOLERANCE

Hanna Pitkänen

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Medicine, University of Helsinki, for public examination in Auditorium 1, Biomedicum 1, on the 23rd of November 2018, at 12 noon.

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Cover art by Kyösti Pitkänen, printed with permission

ISBN 978-951-51-4560-4 (paperback)
ISBN 978-951-51-4561-1 (online)
ISSN 2342-3161 (paperback)
ISSN 2342-317X (online)
Hansaprint
Turenki 2018

To every stray cat in the world:

Olen avaruuksien kissa, olen kissa ihmeellinen.

Olen unien, tähtien kissa, tulin takaa pilvien.

Minä rakensin teille talon, jossa paljon rakastetaan.

Nyt takaisin lähden, mutta palaan uniinne toisinaan.

Kaarina Helakisa, Taivaskissa

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals (I-III)

I Pitkänen H, Jouppila A, Mowinckel MC, Lemponen M, Patiwael S, Sandset PM, Lassila R, Brinkman HJ. Enhanced thrombin generation and reduced intact protein S in processed solvent detergent plasma. Thrombosis Research 2015 Jan;135: 167-74. doi: 10.1016/j.thromres.2014.10.020.

II Pitkänen HH, Jouppila A, Lemponen M, Ilmakunnas M, Ahonen J, Lassila R. Factor XIII deficiency enhances thrombin generation due to impaired fibrin polymerization - An effect corrected by Factor XIII replacement. Thrombosis Research 2017 Jan;149: 56-61. doi: 10.1016/j.thromres.2016.11.012.

III Pitkänen HH, Kärki M, Niinikoski H, Tanner L, Näntö-Salonen K, Pikta M, Kopatz WF, Zuurveld M, Meijers JCM, Brinkman HJM, and Lassila R. Abnormal coagulation and enhanced fibrinolysis due to lysinuric protein intolerance associates with bleeds and renal impairment. Haemophilia. 2018 Aug 2. doi: 10.1111/hae.13543.

ABSTRACT

Background and Aims

Hemostasis is a crucial biological process. Thrombin generation (TG) and fibrin formation are regulated by multiple direct and indirect feedback loops to produce a blood clot. Equally important is the capability to dissolve blood clots through fibrinolysis to restore and repair blood flow. This thesis seeks to recognize those factors that influence TG and fibrinolysis related to solvent/detergent (SD) plasma, congenital FXIII deficiency and lysinuric protein intolerance (LPI). The second aim is to combine traditional and functional coagulation assays to evaluate clot stability. Finally, we aim to characterise the bleeding disorder related to LPI, a metabolic disorder that impairs the transport of cationic amino acids and leads to renal insufficiency. These studies help in designing novel approaches of transfusion therapy in LPI.

Methods

In Study I with experimental *in vitro* study design, several human plasma preparations were used to elucidate the impact of the SD method on TG and clot stability. In Study II both plasma-derived and recombinant factor XIII (FXIII) concentrate therapies were monitored in congenital FXIII deficiency. In Study III the hemostatic and fibrinolytic capacity of 15 LPI patients was investigated. Basic coagulation screen, pro- and anticoagulant factors and thromboelastography or rotational thromboelastometry were performed (I-III). Primary hemostasis was evaluated with PFA-100® (III). *In vitro* TG was assessed using a calibrated automated thrombogram (CAT®) (I-III), and *in vivo* TG was investigated with circulating prothrombin fragments F1+2 (II-III). Gly-Pro-Arg-Pro (GPRP) peptide was used to inhibit fibrinogen polymerization (II). D-dimer (II-III) and plasmin–α2-antiplasmin complex (PAP) (III) were identified as markers of fibrinolysis. Optical density derivatives were assessed to monitor fibrin formation and fibrin degradation (III).

Results and Conclusions

Combining traditional coagulation screening with functional coagulation assays revealed increased fibrinolysis in SD plasma (I), while augmented fibrinolysis could be excluded, even at trough FXIII levels in congenital FXIII deficiency (II). However, increased clot strength was discovered as a response to FXIII concentrate administration. In LPI OD derivatives confirmed fibrinolysis, which was detected with elevated D-dimer levels (III). SD plasma showed enhanced TG and fibrinolysis which could be explained by decreased single chain protein S and α2-antiplasmin

(α2-AP) activity. During SD treatment, the levels of single chain protein S were significantly reduced, leading to a loss of both activated protein C -dependent and -independent protein S activity and enhanced TG. In addition, the *in vitro* coagulation phenotype of SD plasma was even further altered by increased fibrinolysis, possibly caused by the diminished levels of α2-AP. Tranexamic acid abolished fibrinolysis *in vitro* (I).

Low, but hemostatically sufficient, trough levels of FXIII induced accelerated prothrombin conversion, leading to increased TG, possibly based on decreased fibrin antithrombin I (AT I)–like activity (II). According to AT I theory, forming fibrin (= AT I) inhibits TG during the coagulation process by sequestering thrombin. This theory has been somewhat ignored lately, but in our study, impaired fibrin formation induced by the lack of FXIII and GPRP administration led to enhanced TG, thereby supporting the AT I hypothesis (II).

In addition, this thesis aims at determining the pathophysiology behind the bleeding tendency in LPI with various degrees of renal impairment and metabolic disorders. We discovered an impaired primary hemostasis and altered TG. Moreover, we detected reduced fibrin formation and markedly enhanced fibrinolysis that both related to an altered plasminogen/α2-AP ratio and renal insufficiency. In LPI platelet levels, function of primary hemostasis, fibrinogen and FXIII levels should be measured perioperatively and treated accordingly with platelet transfusions and fibrinogen and FXIII concentrates (III).

ABBREVIATIONS

α2-AP	α2-antiplasmin
α2M	α2-macroglobulin
ADP	Adenosine-5-diphosphate
ALT	Alanine aminotransferase
APC	Activated protein C
aPTT	Activated partial thromboplastin time
ARs	Adverse reactions
AT	Antithrombin
BE	Base excess
BS	Bleeding score
CAT®	Calibrated Automated Thrombogram
CLT	Clot lysis time
Crea	Creatinine
CT	Clotting time
DIC	Disseminated intravascular coagulation
eGFR	Estimated glomerular filtration rate
EPI	Epinephrine
ETP	Endogenous thrombin potential
F	Coagulation factor
Fa	Activated coagulation factor
FDP	Fibrin degradation products
FFP	Fresh frozen plasma
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
GAG	Glycosaminoglycans
Gp	Glycoprotein
GPRP	Gly-Pro-Arg-Pro peptide
HCII	Heparin cofactor II
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
LG®	Ligand gel
LPI	Lysiuric protein intolerance
LRP1	LDL receptor related-protein 1
LYS	Lysine
MCF	Maximal clot firmness
ML	Clot lysis

MT	Massive transfusion
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrofil extracellular traps
NO	Nitric oxide
OD	Optical density
P	Plasma
PAI	Plasminogen activator inhibitor
PAP	Plasmin–α2AP complex
PAR	Protease activated receptors
pd	Plasma-derive
PET	Positron emission tomography
PFA-100®	Platelet function analyzer
PL	Phospholipids
POC	Point of care
PPP	Platelet poor plasma
PT	Prothrombin time
r	Recombinant
RBC	Red blood cells
ROTEM®	Rotational thromboelastometry
SD	Solvent/detergent
TAFI	Thrombin activatable fibrinolysis inhibitor
TAT	Thrombin–antithrombin complex
TEG®	Thromboelastography
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TG	Thrombin generation
TM	Thrombomodulin
tPA	Tissue-type plasminogen activator
TPE	Therapeutic plasma exchange
TT	Thrombin time
TXA	Tranexamic acid
U	Urine
uPA	Urokinase-type plasminogen activator
VLDL	Very low-density lipoprotein
VWF	von Willebrand factor

1 INTRODUCTION

Thrombin generation, fibrin formation, and fibrinolysis are essential components for adequate and controlled blood coagulation (Al Dieri et al., 2012; Kolev et al., 2016; Mosesson, 2005). During severe bleeds, the goal is to provide building blocks for sufficient hemostasis and to avoid coagulopathy and pathophysiological thrombus formation (Kozek-Langenecker et al., 2017a). However, what do we measure in order to analyze coagulation markers? Blood samples are collected in tubes containing anticoagulants, most often in citrate, and the blood vessel wall interactions between the components of the blood and the endothelium are lost. Moreover, rheological aspects of coagulation under different flow conditions are almost certainly obscured (Mann et al., 2009). Although combining traditional and functional coagulation screening methods may not provide the whole answer, they can provide a broader valuable view of the process of coagulation.

Congenital coagulation factor deficiencies and component therapies allow us to scrutinize the impact of different coagulation agents on hemostasis and thrombosis. The coagulation system interacts with, and is influenced by, for example, lipids, renal and hepatic insufficiency, and metabolic disorders such as lysinuric protein intolerance (Blasi, 2015; Hiraga et al., 1996; Kayanoki et al., 1999; Madhusudhan et al., 2016). All of these aspects and conditions must be taken into consideration when planning perioperative care for individuals with a bleeding tendency in order to deliver the most efficient and safe treatment.

In addition to combine traditional and functional coagulation assays to investigate clot stability, this study aimed to recognize the factors leading to altered thrombin generation, fibrin formation, and fibrinolysis during administration of solvent/detergent plasma, in congenital factor XIII deficiency, and in lysinuric protein intolerance (LPI). Further, our aim was to characterize the bleeding tendency that relates to LPI, and to design perioperative transfusion approaches in LPI.

2 REVIEW OF THE LITERATURE

2.1 Overview of the Coagulation Cascade

2.1.1 Primary hemostasis

Platelet activation and blood coagulation are mutually dependent interactive processes. Platelets interact with several coagulation factors, and the major player in the coagulation cascade, thrombin, is a potent platelet-activating agonist (Heemskerk et al., 2002).

When circulating platelets encounter vessel wall injury, primary hemostasis is activated. Platelets initially attracted to the site of injury due to interaction of the von Willebrand factor (VWF), provided by endothelial cells and the platelets themselves, and the glycoprotein Ibα (GpIbα) receptor, located on the platelet surface. The next phase is intracellular signaling, leading to platelet activation and conformational change of the platelet receptors and their ligands as well (Reininger, 2008). Collagen receptors are recruited, and phosphatidylserine, a coagulation accelerator, presents itself on the outer platelet membrane as a result of enzymatic activity which translocates it from the inner lipid layer. These activated phospholipid (PL) membranes then assemble and activate the coagulation cascade components, such as the tissue factor (TF)–factor VII (FVII) complex and prothrombinase complex (Butenas et al., 2001). However, since the VWF–GpIbα bond dissociates fast, other receptors, such as GpVI and integrin αIIbβ3, will provide irreversible platelet adhesion and aggregation (Ruggeri et al., 2004), which is ultimately stabilized by the fibrin network within the platelet plug on its surface.

2.1.2 Intrinsic, extrinsic, and common coagulation pathways

The coagulation cascade has been traditionally divided into intrinsic and extrinsic pathways, both of which converge on factor X (FX) activation. Although useful for understanding *in vitro* coagulation tests, this classical theory fails to incorporate the central role of the cell-based surfaces in the *in vivo* coagulation process. The intrinsic pathway (Fig 1) is initiated when contact is made between blood and exposed negatively charged surfaces. The extrinsic pathway (Fig 1) is activated by TF, expressed by subendothelial tissue when vascular trauma occurs (Lind et al., 2003).

Coagulation can also be regarded as a time-based process involving the following four steps: initiation, amplification, propagation, and stabilization. According to this model, coagulation is initiated mainly by FVIIa-bound TF, which then activates initial prothrombin activation by FXa-Va. In the amplification phase, thrombin activates FV, FVIII, and FXI to produce more thrombin via a positive feedback loop. During propagation, continuation of thrombin formation is mainly produced by the ongoing generation of FXa by FIXa and FVIIIa. Fibrin formation induces maximum thrombin generation (TG). When thrombin cleaves fibrinogen to fibrin, thrombin is adsorbed to the fibrin. Fibrin–bound thrombin then further enhances fibrin formation by activating FV and FVIII and the platelets. Further still, thrombus-associated thrombin is resistant to antithrombin activity (Weitz et al., 1990). At the very end of the coagulation cascade, thrombin and Ca^{2+} catalyze the formation of FXIIIa, which then stabilizes the fibrin clot by crosslinking the deposited fibrin fibrils (Bombeli et al., 2004).

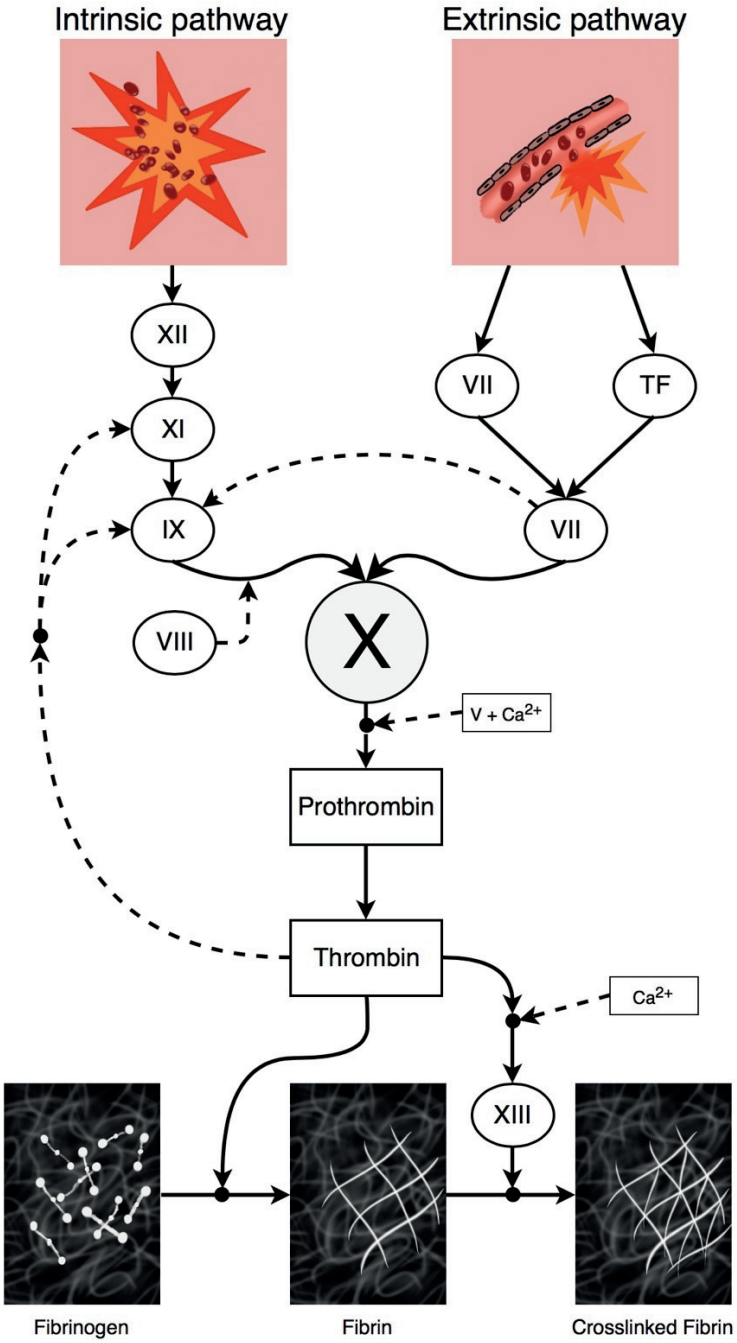

Figure 1. *Schematic view of a classical coagulation cascade. Ca^{2+} = calcium ion; TF = tissue factor. Illustration by Heikki Valkonen.*

2.1.3 Protein C pathway and single chain protein S

The protein C anticoagulant pathway serves as a major system for controlling thrombosis. Thrombomodulin (TM), a thrombin binding protein located on the surface of the endothelial cells, forms a high affinity complex with thrombin. Although thrombin can activate protein C on its own, formation of the thrombin–TM complex accelerates the formation of both the thrombin activatable fibrinolysis inhibitor (TAFI) (Bajzar et al., 1996) and the activated protein C (APC) (Esmon et al., 1981). APC then forms a complex with protein S to inactivate FVa and FVIIIa (Walker, 1980, 1981). In addition, protein S is a tissue factor pathway inhibitor (TFPI) cofactor and participates in the inhibition of the TF/FVIIa/FXa complex (Hackeng et al., 2009). Protein S may also present direct anticoagulation activity by inhibiting the assembling and activation of FVIIIa/FIXa and FVa/FXa complexes (Heeb et al., 2009; Takeyama et al., 2008). Protein S embodies a thrombin-sensitive region that is susceptible to proteolytic cleavage by FXa, thrombin, and elastase. When cleaved, the capability of protein S to function as an APC cofactor is lost. Whether the same impairment is true for the direct anticoagulant activity and the TFPI-cofactor activity remains unknown (Brinkman et al., 2005; Heeb et al., 2002; Long et al., 1998; Morboeuf et al., 2000). In plasma, both an active single chain molecule and an inactive two chain form of protein S can be detected as a result of the cleavage at Arg60 (Dienava-Verdoold et al., 2012).

2.2 Coagulation and Lipids

2.2.1 Impacts of the lipid profile on coagulation balance

Coagulation *in vivo* is a result of the interplay between cells, membranes, blood vessels, and coagulation factors, inhibitors and cofactors (Solum, 1999) under the conditions of blood flow. Phospholipids present the surface structures for coagulation cascade and remarkably accelerate these reactions. For example, TF is an integral membrane protein that interacts with phospholipids, and the plasma membranes of activated platelets provide a catalytic surface for the factor IXa–VIIIa (tenase) complex, which leads to the activation of FXa. Factor Xa–Va complex (prothrombinase) uses the same membrane structures for a platform of prothrombin conversion to thrombin (Bouchard et al., 1997).

In addition to phospholipids, lipoproteins and triglycerides also influence the coagulation system. Low-density lipoprotein (LDL), the major cholesterole transporter in humans, is prone to oxidation and considered a risk factor for developing coronary artery disease (Berliner et al., 1996). The human very low-

density lipoprotein (VLDL) in plasma is very rich in triglycerides and delivers free fatty acids to adipose tissue (Merkel et al., 2002). Hypertriglyceridemia, but not hyperlipoproteinemia, is associated with elevated levels of tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1), leading to alterations of the fibrinolytic system (Hiraga et al., 1996). VLDL is known to support the prothrombinase complex, and there is a positive correlation between triglyceride concentrations and vitamin K-dependent coagulation factors (FII, FVII, FIX and FX) (Moyer et al., 1998). Furthermore, VLDL has the capability to prime TG via prothrombinase complex support (Rota et al., 1998), and protein S has been identified in its proteome (Dashty et al., 2014). Moreover, both LDL and VLDL are known to carry the natural anticoagulant TFPI (Wesselschmidt et al., 1992). In contrast to LDL and VLDL, high-density lipoprotein (HDL) solely exhibits anticoagulant activities as a cofactor of proteins C and S (Griffin et al., 1999).

Intriguingly, lipoprotein(a), a molecule formed by an LDL particle bound to a glycoprotein called apolipoprotein(a) (Utermann 2006), provides yet another link between the coagulation system and lipids. Lipoprotein(a) has structural similarities with plasminogen and it has been shown to competitively inhibit plasminogen activation and promote the formation of stenosis in blood vessel walls, expressing both antifibrinolytic and atherosclerotic capacity (Hancock et al., 2003; Kamstrup et al., 2012).

2.2.2 Low-density lipoprotein receptor-related protein 1

The LDL receptor related-protein 1 (LRP1) is a large endocytic receptor that is expressed in several tissues. It has a wide variety of structurally unrelated ligands. LRP1 mediates the hepatic removal and degradation of proteinase–inhibitor complexes, activated coagulation factors, and chylomicron remnants (Lillis et al., 2008). LRP1 is able to down-regulate coagulation by removing the TFPI–VIIa complex, FVIII and FIXa from plasma, although the FVIII bound to VWF is protected from clearance (Hamik et al., 1999; Lenting et al., 1999; Neels et al., 2000; Saenko et al., 1999). In addition, LRP1 has a high affinity for some protease-inhibitor complexes, including the thrombin inhibitor α2-macroglobulin–protease complex, urokinase-type plasminogen activator–plasminogen activator inhibitor type 1 (uPA–PAI-1) complex, and thrombin–PAI-1 complex (Stefansson et al., 1996; Strickland et al., 1990).

2.3 Thrombin

2.3.1 Structure

Adequate hemostasis is crucially dependent on sufficient thrombin formation since thrombin is the key element in converting fibrinogen into a fibrin network and stimulating platelets to form a hemostatic plug. Thrombin plays a pivotal role in clot promotion, activation of its regulation, and inhibition of fibrinolysis (Al Dieri et al., 2012; Spronk et al., 2003).

Thrombin belongs to a family of chymotrypsin serine proteases, which have a serine residue in the active site cleft. Exposed hydrophobic loops (60- and γ-loop) and charged patches called exosites surround the active site (Bode, 2005). Another important feature of thrombin is the loop containing the Na^+-binding site. The binding of the sodium ion allosterically modulates the activity of thrombin and increases the access of small procoagulant substrates (i.e., fibrinogen, FV, FVIII, PAR1) over anticoagulant ones (i.e., protein C) (Page et al., 2005). However, given a highly regulated plasma sodium ion concentration, sodium is not regarded as a true thrombin cofactor (Adams et al., 2006).

2.3.2 Exosites

Thrombin and other coagulation proteinases hold a marked and distinct specificity for their substrates. Circulating levels of coagulation enzyme and substrate are typically in nanomolar levels, with the exception of fibrin, antithrombin (AT) and prothrombin having micromolar concentrations, and require effective recognition and binding for the coagulation process to proceed (Mann et al., 2003). Despite extensive investigations, the molecular base for such specificity remains unknown, but the exosite-driven enzymatic function may offer a plausible explanation (Krishnaswamy, 2005). Thrombin exocites I and II are located on the opposite sides of the active site. Exosite I is a binding epitope for fibrinogen, fibrin, TM, and the protease-activated receptors, PAR1 and PAR4 (Ayala et al., 2001; Pineda et al., 2002). Exosite II forms the site of interaction with heparin and other glycosaminoglycans that are required for protease inactivation by serpins (Gettins, 2002; Olson et al., 2002). Further, exosite II provides a platform for interaction with the platelet receptors, GpIbα and FVIII (De Cristofaro et al., 2000; Nogami et al., 2005).

2.3.3 Substrates and regulators

Thrombin is able to cleave to at least 12 substrates and has 5 cofactors, if the sodium ion is included. Figure 2 summarizes some of the main thrombin substrates, cofactors and regulators.

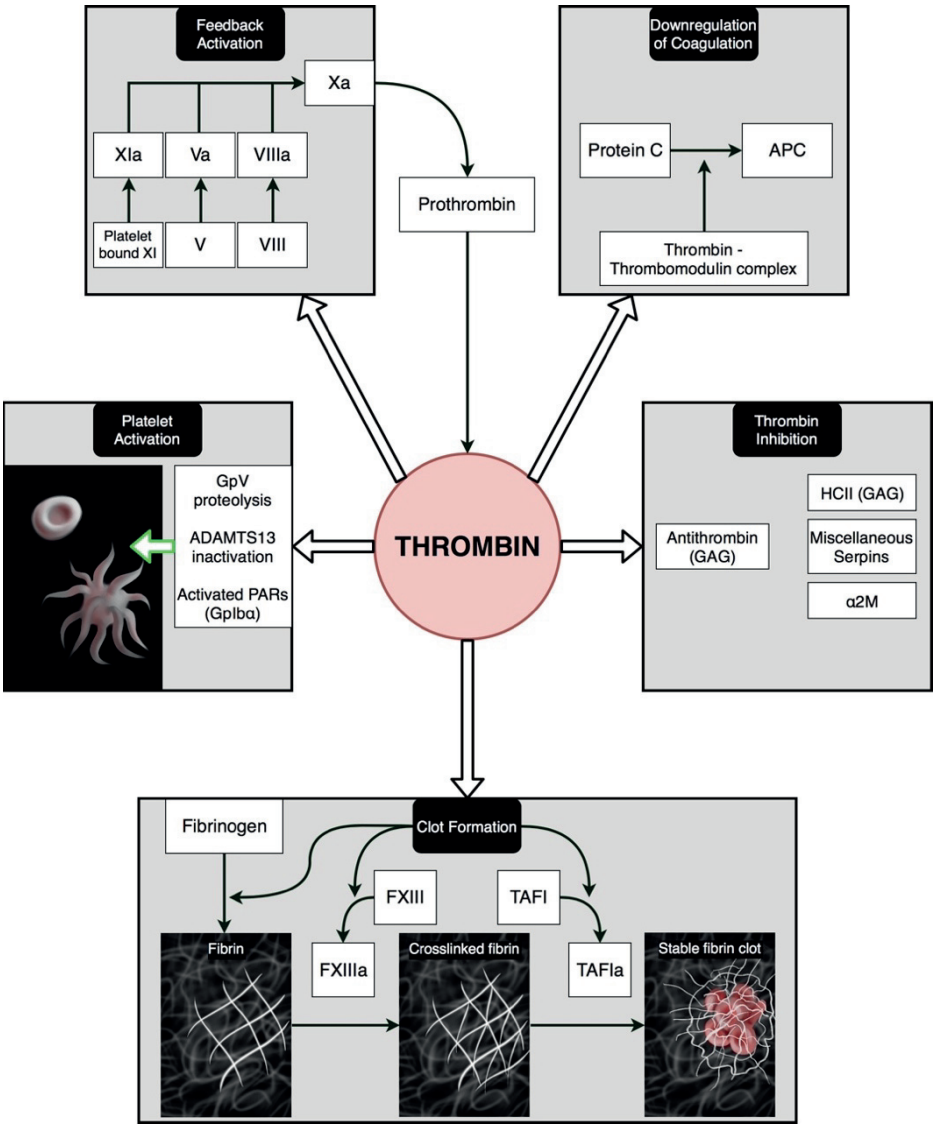

***Figure 2**. Summary of thrombin substrates, cofactors (in parentheses) and regulators, adapted from Adams & Huntington and Crawley et al. (Adams et al., 2006; Crawley et al., 2007). α2M = α2-macroglobulin; APC = activated protein C; GAG = glycosaminoglycans; GpIbα = glycoprotein Ibα; GpV = glycoprotein V; HCII = heparin cofactor II; PARs = protease-activated receptors; TAFI = thrombin-activatable fibrinolysis inhibitor. Illustration by Heikki Valkonen.*

2.3.4 Thrombin generation

The 37-kDa thrombin is generated from its zymogen prothrombin by proteolytic cleavage by factor Xa. Previous studies have shown that an increase in TG associates with a prothrombotic phenotype (Ay et al., 2011; Curvers et al., 2002; Kyrle et al., 1998), whereas decreased TG can produce a bleeding tendency (Beltran-Miranda et al., 2005; Ten Cate, 2012). The net result of TG is determined by prothrombin conversion and thrombin inactivation (Kremers et al., 2015). Enhanced TG can be induced by an increase of prothrombin (Dielis et al., 2008), overactive prothrombin conversion (Castoldi et al., 2004; Duchemin et al., 2008), or decreased thrombin breakdown (Sniecinski et al., 2008). Decreased TG can be caused by inadequate prothrombin conversion and thrombin inactivation or both (Hemker et al., 2013). The main thrombin inhibitors in plasma are AT, α2-macroglobulin (α2M), and heparin cofactor II (HCII) and to a lesser extent, the group of miscellaneous serpins (α1-antitrypsin, α1-proteinase inhibitor, α1-antichymotrypsin, protease Nexin 1, C1 inhibitor and others) (Adams et al., 2006; Kremers et al., 2017; Pratt et al., 1989). In addition, plasma fibrin(ogen) levels affect thrombin inactivation, although the underlying mechanism still remains unclear (Kremers et al., 2014).

2.3.5 Thrombin generation assays - Calibrated Automated Thrombogram

The thrombin generation curve provides an old and essential tool in coagulation research. Increasing evidence shows that the amount of thrombin activity that develops is a better marker of the function of the clotting system than the time it takes for clotting to start (= clotting time) (Al Dieri et al., 2012). *"The more thrombin, the less bleeding, but the more thrombosis, the less thrombin, the more bleeding, but the less thrombosis"* –First Law of Hemostasis and Thrombosis (Hemker, 2015).

Calibrated automated thrombogram (CAT®) displays a concentration of thrombin in clotting plasma with or without platelets by monitoring the splitting of a slow-reacting fluorogenic substrate and comparing it to a constant known thrombin activity in a parallel, non-clotting sample. Coagulation is activated by small amounts of TF and phospholipids. Software has been developed to combine the data from the two experiments to calculate thrombin concentration in the coagulating sample in real time (Hemker et al., 2002, 2003). The main variables of the CAT® curve are lag time, endogenous thrombin potential (ETP) measured as the area under the curve, and the peak as illustrated in Figure 3. Additional variables include time to peak and steepness of the rising slope. The lag time can be considered the clotting time. The ETP is the most robust and important variable since it is determined from the extent of the fluorogenic product being produced by free thrombin. The peak is

more sensitive because it derives from the first derivative of the product–time curve (Tripodi, 2016).

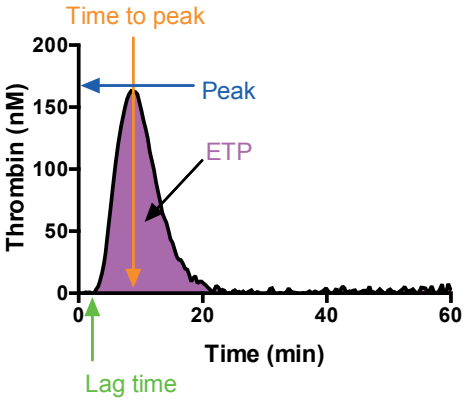

Figure 3. *CAT® variables illustrated in thrombin generation curve. Lag time (time from initiation of the reaction to the start of thrombin formation), ETP (endogenous thrombin potential) described as the area under the curve, peak thrombin generation and time to peak.*

2.4 Fibrinogen

Fibrinogen is a plasma glycoprotein synthesized by the liver with a molecular weight of 340-kDa. Fibrinogen can be measured with the Clauss method performed by adding a high concentration of thrombin to dilute test plasma and clotting time is measured. The test result is compared with a calibration curve prepared by clotting a series of dilutions of a reference plasma sample of known fibrinogen concentration, and a result in g/L is obtained (Mackie et al., 2003). Human plasma usually contains around 1.5–3.5 g/L of soluble fibrinogen, which plays an important role in clot formation and platelet aggregation. The roles of fibrinogen and fibrin intersect in blood clotting, fibrinolysis, cellular and matrix interactions, the inflammatory response, wound healing, and neoplasia (Mosesson, 2005). Fibrinogen molecules are elongated 45 nm structures that are comprised of two sets of disulfide-bridged Aα-, Bα- and γ-chains, which are joined together within the N-terminal E domain. Each molecule consists of two outer D domains, each connected by a coiled-coil segment to its central E domain (Henschen et al., 1983; Huang et al., 1993). Thrombin catalyzes the fibrinogen conversion to fibrin. Thrombin–fibrinogen substrate binding results in cleavage and the release of N-terminal fibrinopeptide A (FPA) and finally fibrinopeptide B (FPB).

The resulting polymerized fibrin possesses a residual non-substrate, thrombin-binding potential termed antithrombin I (AT I). TG down-regulating AT I–like activity can be defined as the adsorption of thrombin to fibrin during the coagulation process leading to fibrin–bound thrombin (Mosesson, 2007; Seegers et al., 1954). AT I is determined by two classes of non-substrate thrombin-binding sites in fibrin(ogen) D and E domains containing a γ-chain (Meh et al., 1996). Congenital a-, dys- and hypofibrinogemia may render patients susceptible to either bleeds or thromboembolic disease, and their bleeding phenotypes correlate strongly with the basal fibrinogen levels. However, the pathogenesis of thrombus formation in these congenital conditions is unknown, but patients are able to generate thrombin, both in the initial phase of limited production and in the secondary burst of thrombin generation even in the absence of fibrinogen (de Moerloose et al., 2010). Prothrombin activation and TG are eminent in afibrinogenemic plasma based on elevated prothrombin F1+2 fragments and thrombin–antithrombin complexes (TAT). These markers are normalized by fibrinogen infusion, suggesting that the absence of fibrin and its AT I–like effect leads to increased levels of circulating thrombin (de Bosch et al., 2002; Dupuy et al., 2001). This enhancement implies that thrombin, by binding to fibrin in clotting blood, significantly suppresses TG (Mosesson, 2005).

2.5 Fibrin Formation

Thrombin-induced cleavage of fibrinopeptides from fibrinogen exposes binding sites in the central nodule, complementary to sites constitutively available at the ends of the molecule. As a result, fibrin polymerization is initiated, and fibrin monomers are produced. Fibrin monomers assemble into fibrin polymers in a spontaneous, ordered process, where the FPA release initiates protofibril formation and an FPB release enhances lateral aggregation (Weisel et al., 1993). In the next step of fibrin formation, the thrombin-activated factor XIII (FXIIIa) converts fibrin polymers into insoluble fibers by catalyzing the formation of covalent intramolecular bonds between the α- and γ-chain lysine and glutamine residues in a reaction called crosslinking (Kanaide et al., 1975; Lorand, 1972). FXIIIa also crosslinks α2-antiplasmin (α2-AP), TAFI and PAI-1, 2 and 3 to fibrin, rendering it resistant to fibrinolysis (Ritchie et al., 2000, 2001).

2.6 Factor XIII

The coagulation factor XIII (FXIII) is a protransglutaminase activated into its tranglutaminase form by thrombin and Ca^{2+} in the final stages of the coagulation cascade. FXIII is a heterotetramer (FXIII-A_2B_2) that consists of two active FXIII-A_2 subunits and two protective/inhibitory FXIII-B_2 subunits that prolong the lifespan of FXIII-A in the circulation. Thrombin first cleaves off an activation peptide from FXIII-A, and secondly, FXIII-B dissociates from the heterotetramer in the presence of Ca^{2+}, finalizing the formation of activated FXIII (FXIIIa) (Komáromi et al., 2011; Muszbek et al., 2011). FXIII-A originates from bone marrow, whereas FXIII-B is produced by hepatocytes. FXIII-A_2, but not FXIII-B_2, can be found in the cytoplasm of platelets and monocytes/macrophages (Adány et al., 1987; Nagy et al., 1986; Poon et al., 1989). The main function of FXIII is to crosslink fibrin chains and the fibrinolysis inhibitors to fibrin as described above. In addition to coagulation, FXIII has multiple another intra- and extracellular functions, namely, maintaining pregnancy, wound healing, and osteosynthesis (Inbal et al., 2005; Muszbek et al., 2011).

Congenital FXIII deficiency is a severe coagulation disorder inherited as an autosomal recessive trait with an incidence of 1:2–3 million. Most cases involve FXIII-A deficiency, while FXIII-B deficiency rarely causes a significant bleeding tendency due to the short lifespan for circulating FXIII-A (Komáromi et al., 2011). Patients with FXIII deficiency are prone to bleeds from the umbilical cord after birth, prolonged postoperative bleeding, poor wound healing, recurrent spontaneous miscarriage, bleeds in soft and subcutaneous tissue, and the central nervous system (Lassila, 2016; Muszbek et al., 2011). FXIII deficiency can be treated with fresh frozen plasma (FFP), cryoprecipitate, and plasma-derived virally inactivated FXIII concentrate (pdFXIII) (Gootenberg, 1998). Also, a new recombinant FXIII concentrate (rFXIII) is now available for FXIII-A subunit deficiency. rFXIII is capable of forming a stable heterotetramer with endogenous FXIII-B (Carcao et al., 2017; Inbal et al., 2012). FXIII concentrate therapy is an efficient monthly prophylactic treatment for FXIII deficiency. According to a recent study, the trough level for prophylaxis can be set to a FXIII:C of 15 %, which is achievable by the administration of 25 to 35 IU/kg every 4 to 6 weeks (Menegatti et al., 2017).

2.7 Fibrin clot stability

Various genetic and environmental factors modulate the formation of a fibrin clot and boost its resistance to fibrinolysis. These factors include fibrinogen and FXIII polymorphism, elevated levels of prothrombin, thrombin, and homocysteine (Scott et al., 2004). Higher concentrations of (pro)thrombin produce thinner and more tightly packed fibrin fibers with smaller pores (Wolberg et al., 2003). Also, different disease states, such as that of diabetes, hyperlipidemia in the form of high LDL cholesterol and triglyceride levels, hypertension, infection, and inflammation produce less lysable clots. Moreover, fibrin architecture can be modulated by drugs like acetylsalicylic acid, statins, glucose-lowering agents and anticoagulants (Undas et al., 2011). For example, simvastatin therapy for hypercholesterolemia decreases clotting by inhibiting the activation of prothrombin, FV, and FXIII, and increases the inactivation of FVa, without affecting actual fibrinogen levels in plasma (Undas et al., 2001).

2.8 Fibrinolysis

Fibrinolysis occurs when plasmin binds to its substrate, fibrin, on a clot surface. The players and regulators' participating in fibrinolysis are well established (Longstaff et al., 2015), as shown in Figure 4. Initially, inactive proenzyme plasminogen is converted to plasmin on fibrin's surface by tPA or urokinase-type plasminogen activator (uPA) (Rijken et al., 1981). The PAI family and serin protease Nexin are able to inhibit both tPA and uPA (Thelwell et al., 2007). Besides PAI-1, α2-AP is another crucial and very potent member of the serpin inhibitors of fibrinolysis. α2-AP is the primary plasmin(ogen) inhibitor in humans. It regulates fibrinolysis in three ways: by forming a complex with plasmin, by inhibiting adsorption of plasminogen to fibrin, and by reinforcing fibrin against local plasmin effect via FXIII–mediated crosslinkage. However, the antifibrinolytic activity of α2-AP can be slowed down by plasmin bound to fibrin or lysine analogues. Other serpins with antifibrinolytic capacity include PAI-2, PAI-3 and α2M, whereas TAFI can be characterized as a fibrinolysis inhibitor within a clot. TAFI is activated by thrombin–TM complex or plasmin during ongoing fibrinolysis, and inhibits fibrinolysis by reducing plasminogen and plasmin binding to fibrin surfaces (Longstaff et al., 2015; Rijken et al., 2009).

Fibrinolysis is also affected by clot architecture. Red blood cells are trapped in a fibrin network via FXIIIa-mediated clot retention, leading to resistant clots for fibrinolysis (Aleman et al., 2014). Intriguingly, plasmin is able to counterbalance the

situation by inactivating FXIIIa (Hur et al., 2015). Activated neutrofils release DNA and histones at the sites of intravascular thrombi. These neutrofil extracellular traps (NETs) are capable of delaying fibrinolysis by holding the lysing fibrin strands together (Longstaff et al., 2013).

Ongoing hyperfibrinolysis can be detected by increased fibrin degradation products (FDPs), plasmin–α2AP complex (PAP), tPA, and TAFIa (Longstaff, 2018). FDPs are the end product of the fibrin degradation by plasmin. Digestion of crosslinked fibrin by plasmin yields large X-oligomer fragments and D-dimers with two D domains from the adjacent fibrin molecules held together by remnants of the γ-chain bond. D-dimer is regarded as a specific marker of the FXIII-crosslinked fibrin degradation products. Degradation of fibrinogen and non-crosslinked fibrin also produces FDPs, but their clinical significance is presumed to be less important (Gaffney, 2001). Besides FDPs, fibrinolysis can be investigated, for example, in plasma via clot lysis methods or in whole blood by viscoelastic applications (Longstaff, 2018).

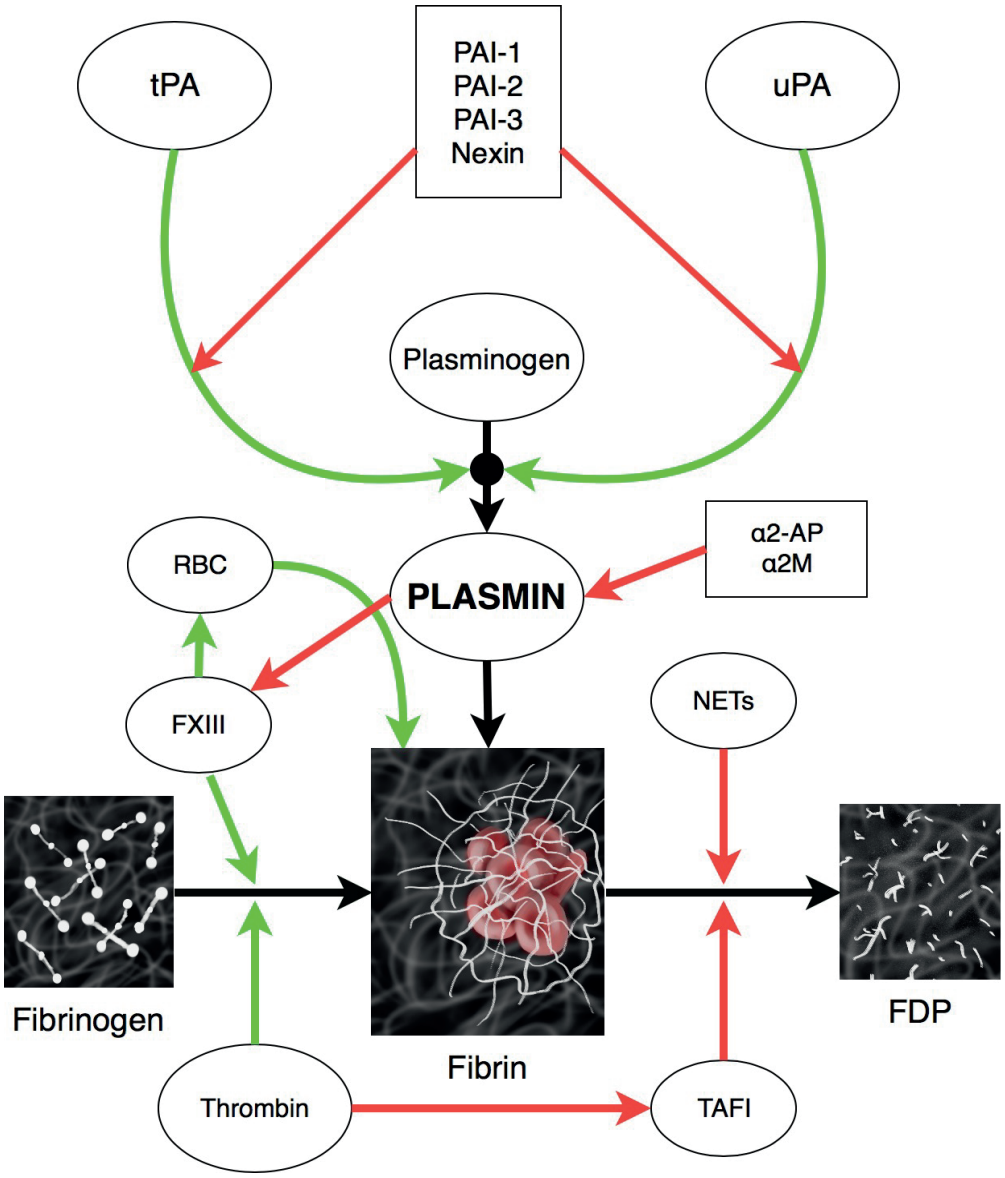

***Figure 4**. Fibrinolysis activators and inhibitors, adapted from Rijken and Lijnen (Rijken et al., 2009). Activators are in green and inhibitors are in red. α2-AP = α2-antiplasmin; α2M = α2-macroglobulin; FDP = fibrin degradation products; FXIII = coagulation factor XIII; NETs = neutrophil extracellular traps; PAI = plasminogen activator inhibitor; RBC = red blood cells; tPA = tissue-type plasminogen activator; uPA = urokinase-type plasminogen activator; TAFI = thrombin activatable fibrinolysis inhibitor. Illustration by Heikki Valkonen.*

2.9 Lysinuric protein intolerance

Lysinuric protein intolerance (LPI) is a rare recessive autosomal disorder that impairs the transport of the cationic amino acids lysine, ornithine, and arginine in the basolateral membrane of the intestine, lung, liver, and renal tubules leading to decreased intestinal absorption and the excessive renal loss of these amino acids. Supplementation of citrulline partially restores deficiency of ornithine and arginine, but lysine deficiency must be corrected with individual low-dose oral lysine supplementation (Norio et al., 1971; Perheentupa et al., 1965; Rajantie et al., 1980; Tanner et al., 2007a). LPI is caused by biallelic mutations in the gene SLC7A7, which encodes the cationic amino-acids transporter y+LAT-1 protein (Borsani et al., 1999; Torrents et al., 1998). LPI belongs to the Finnish disease heritage and is most prevalent in Finland (1 in 60,000), although numerous other cases have been reported (Koizumi et al., 2000; Sperandeo et al., 2000). All Finnish patients share the same mutation caused by the substitution of a T for an A at the cDNA position 1181-2 (Norio et al., 1971).

The low plasma concentrations of arginine and ornithine lead to hyperammonemia. Long-term complications include renal insufficiency that can lead to end-stage renal disease, pulmonary fibrosis, and alveolar proteinosis, hypercholesterolemia, hypertriglyceridemia, and hematological complications, including hemophagocytic syndrome with strongly elevated ferritin levels (DiRocco et al., 1993; Simell et al., 1975; Tanner et al., 2007b, 2010). LPI patients are prone to bleeds. Fatal hemorrhage has even been observed postpartum and otherwise, but the mechanisms are unknown, although low platelet counts, low fibrinogen, high D-dimer and elevated levels of TAT complex have been observed (Kayanoki et al., 1999; Tanner et al., 2006). Since the pathogenesis of the abnormal coagulation in LPI is unidentified, prophylaxis or treatment algorithms for bleeds have not yet been made available. Due to the severe renal insufficiency of LPI patients, they may require invasive procedures related to renal replacement therapy and renal transplant (Tanner et al., 2007b), rendering them susceptible to bleeds.

Underlying pathophysiological mechanisms of renal insufficiency in LPI are unclear, but suggestions have been made. According to the first hypothesis, lysine trapped inside proximal tubular cells is cytotoxic, and accordingly, the administration of oral lysine in rats causes proteinuria and prevents albumin re-absorption. In addition, lysine can activate apoptosis via the NADPH- (nicotinamide adenine dinucleotide phosphate) dependent mechanism, thereby promoting oxidative stress (Thelle et al., 2006; Verzola et al., 2012). Secondly, a local immune system dysfunction in renal tissue due to the increased levels of plasma nitric oxide

(NO) has been proposed. Besides lysine, intracellular arginine levels are also elevated in LPI. Arginine is a substrate of inducible nitric oxide synthase that is naturally occurring in renal cells. The increased levels of NO trigger inflammation and an immune defense, which might lead to kidney damage (Mannucci et al., 2005; Nagasaka et al., 2009).

2.10 Current Transfusion Therapy

2.10.1 Blood products and plasma fractionation

Blood component therapy includes red blood cells (RBCs), plasma, and platelets. Transfusions save lives during severe bleeding, but it is also associated with complications and thus should be used only when necessary (Kozek-Langenecker et al., 2017b). In the International Hemovigilance Network Database report from 2006 to 2012, gathered from 25 countries covering 132.8 million blood components issued, the incidence of all adverse reactions (ARs) were 77.5 per 100,000 components issued. Of all ARs, 25 % were severe (19.1 per 100,000). The incidence of death was 0.26 per 100,000, with 58 % of deaths caused by transfusion-associated circulatory overload, transfusion-associated acute lung injury and transfusion-associated dyspnea (Politis et al., 2016).

In order to prepare plasma and plasma-derived concentrate products, pooled plasma from whole blood or apheresis products is collected by blood banks, for example, the Finnish Red Cross in Finland and Sanquin in The Netherlands. First, the blood is collected and anticoagulated using an automated process. The components of whole blood are separated by centrifugation into plasma, platelets, and RBCs. The plasma is frozen after the collection at a core temperature of -50 ^{O}C. Plasma can then be fractionated into albumin, coagulation factors, protease inhibitors, and immunoglobulins (Punainen Risti Veripalvelu, Suomen Punainen Risti (28 Dec. 2017). Retrieved 07 July 2018, from http://www.veripalvelu.fi/verenluovutus/veren-matka/valmistetaan).

The solvent/detergent (SD) method is used to inactivate enveloped viruses during plasma protein preparations. Plasma is treated with tri(n-butyl)phosphate and 1 % Triton X-100. In addition to improved viral safety, the SD method also alters the lipid composition of plasma due to delipitation, making it especially poor in LDL and VLDL (Hellstern et al., 2011a). Moreover, the SD method decreases antitrypsin activity by 50 % and completely abolishes α2-AP acitivity likely due to the conformational changes caused by solvent, detergent and heat (Mast et al., 1999).

An additional time-reducing chromatographic prion-binding step, called ligand gel (LG®), has been implemented to the SD treatment (Heger et al., 2009).

2.10.2 Concentrates for replacement therapy of coagulation factors

Plasma-derived and recombinant concentrates are a part of general coagulation management when a severe bleed occurs or for regular prophylaxis mainly for inherited bleeding disorders. A fibrinogen concentrate dose of 25–50 mg/kg should be administered, when the fibrinogen levels in plasma fall below 1.5–2.0 g/L. FXIII activity should be kept above 30 % at least, and FXIII concentrate is given 30 IU/kg in order to maintain adequate levels. Prothrombin complex concentrate can be used as part of goal-directed transfusion therapy and the reversal of oral vitamin K antagonists. Tranexamic acid (TXA) 20–25 mg/kg is recommended when fibrinolysis is detected or suspected. TXA is a synthetic lysine analogue and a competitive inhibitor of plasminogen and plasmin. It inhibits fibrinolysis by competing for the lysine binding sites in plasminogen, thus preventing plasminogen binding to fibrin and its activation on fibrin´s surface (Mannucci et al., 2007). In trauma, TXA reduces mortality when given early within 3 hours of injury, but if administration is delayed beyond this timeframe, the risk of mortality increases. Recombinant FVII is not recommended due to an increased risk for thrombosis, and desmopressin should only be given in special circumstances, such as acquired von Willebrand disease and platelet function impairment in those cases without a risk for thrombosis (Kozek-Langenecker et al., 2017a; Roberts et al., 2017).

2.10.3 Massive transfusion

Massive transfusion (MT) is a term that refers to large volumes of blood products being administered during a short period of time to treat severe or uncontrolled bleeding events (Pham et al., 2013). The generally accepted definitions for MT in adults are shown in Table 1.

Table 1. *Definition of a massive transfusion. Adapted from Pham and Shaz 2013.*

Definition of a Massive Transfusion
1. Transfusion of ≥ 10 red blood cell (RBC) units (roughly the total blood volume of an average adult) within 24 hours
2. Transfusion of > 4 RBC units in 1 hour and an expected need for a continued blood product support, and
3. Restoring > 50 % of TBV with blood products within 3 hours

TBV = total blood volume

In clinical decision-making, it is not always clear when a patient needs an MT. Several models have been studied and published. For trauma, Nunez et al. (2009) developed a simple and efficient scoring system (Table 2) to trigger an MT protocol.

Table 2. *The scoring system used to trigger the massive transfusion (MT) protocol. Two of the four markers must be positive for the initiation of the MT protocol. Adapted from Nunez et al. 2009.*

Massive Transfusion Triggering Protocol
Penetrating trauma
Systolic arterial pressure < 90 mmHg
Heart rate > 120 /min
Positive FAST

FAST = Focused Assessment with Sonography in Trauma

The goal of MT is to maintain adequate arterial blood pressure and oxygen delivery to vital organs during severe bleeding. The current MT protocol consists of RBCs, plasma, and platelets given in a 1:1:1 ratio, which results in a mixture resembling blood. The PROPPR Trial compared transfusion ratios of 2:1:1 and 1:1:1. There was no significant difference between 24-hour or 30-day mortality. Nontheless, more patients in the 1:1:1 group attained hemostasis, and fewer died from exsanguination in 24 hours (Dzik et al., 2011; Holcomb et al., 2015).

2.10.4 Point of care monitoring

Viscoelastic point of care (POC) coagulation devices include thromboelastography (TEG® by the Hemoscope Corporation, Niles, IL, USA) and rotational thromboelastometry (ROTEM®, initially by Pentapharm, now TEM International

GmbH, Munich, Germany) (Theusinger et al., 2015). Traditional coagulation markers, such as activated partial thromboplastin time (aPTT) and prothrombin time (PT) measure the time from the activation of intrinsic and extrinsic coagulation pathways to the initiation of clot formation. In contrast to POC, traditional coagulation markers fail to provide any information of clot formation kinetics, stability, or lysis. Viscoelastic POC devices were thus developed to guide goal-directed transfusion therapy, which aims at reducing the use of blood products, adverse events, and costs. Goal-directed transfusion therapy emphasizes the use of coagulation factor concentrates instead of plasma (Lier et al., 2013). Different POC algorithms have been created for cardiac surgery, trauma, and liver surgery. A recent Cochrane review concludes that although there is growing evidence that supports the use of TEG®- or ROTEM®-guided transfusion strategies, the majority of studies have examined elective cardiac surgery, and further research in acute situations and other patient categories is needed (Wikkelsø et al., 2016).

In both of the viscoelastic POC devices, reagent and citrated whole blood are placed into a cup and incubated at $37^{O}C$. The viscoelastic properties are measured by introducing a pin into the forming blood clot. In TEG®, the cup oscillates and in ROTEM® the pin oscillates, as described in detail in Figure 5 (Luddington, 2005). The terminology for the ROTEM® measurements is explained in Figure 6. ROTEM® allows for real-time visualization of clot formation, and five different tests are commonly used for coagulation monitoring. In EXTEM, coagulation is initiated by TF, leading to extrinsic pathway activation. With INTEM, a contact activator is added to activate the intrinsic coagulation pathway. FIBTEM shows the fibrin clot forming under the influence of FXIII without the impact of platelets that are inactivated by cytochalasin D. Fibrinolysis can be inhibited by adding aprotinin in a test called APTEM, while the influence of heparin can be detected by the addition of heparinase before contact activation in the HEPTEM test (Theusinger et al., 2015). ROTEM® is not able to detect any hemostasis defects caused by pH, Ca^{2+} deficiency, von Willebrand disease, and has poor sensitivity to hemophilia (Bowyer et al., 2013; Lier et al., 2013). The limit of detection in ROTEM® depends on high concentrations of activators that are able to bypass the deficient VWF and FVIII and FIX, at least.

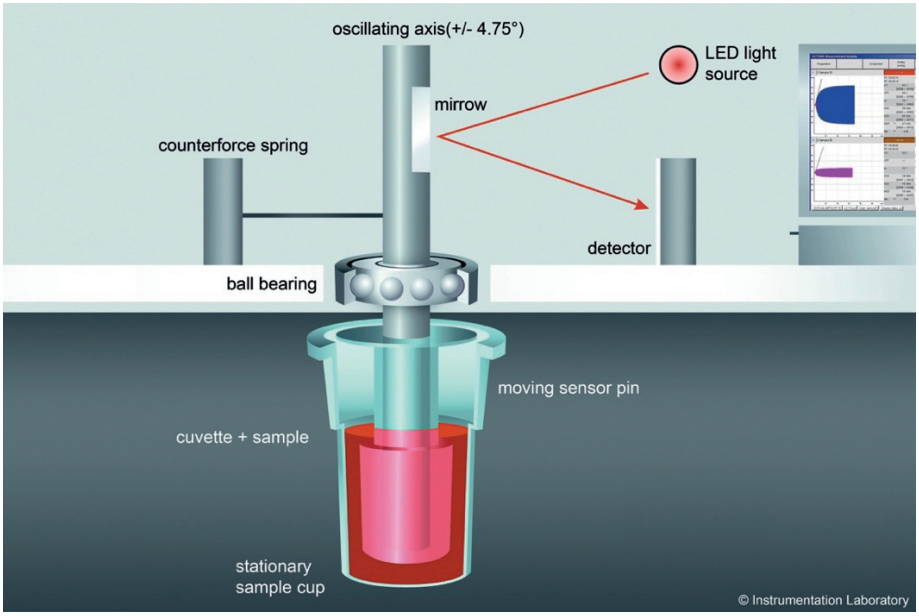

Figure 5. *The ROTEM® measuring principle. Whole blood sample is added to a cup and a pin is immersed. The pin is rotated freely until a clot begins to form and movement is restricted. This change in kinetics is detected mechanically and converted to numerical values by specific software. Figure by TEM® International, reprinted with permission of copyright holder.*

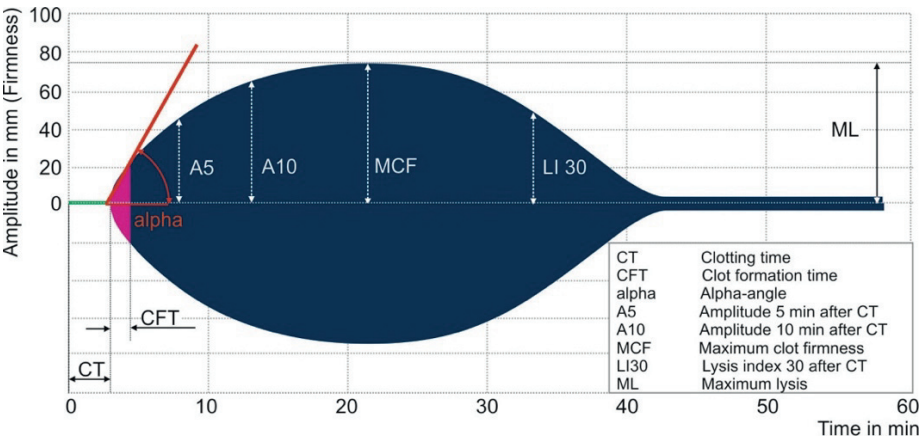

Figure 6. *ROTEM® parameters providing information on the kinetics of hemostasis. Figure by TEM® International, reprinted with permission from the copyright holder.*

3 AIMS OF THE STUDY

During severe bleeds and for congenital coagulation factor deficiencies, the aim of management is to ensure optimal physiological TG and fibrin formation. Traditional coagulation screening provides quantitative data, but it presents only limited details of function and quality. Parallel measurements of traditional coagulation screen, TG, and ROTEM® variables enable functional coagulation assessment. This study seeks to identify the factors that modify TG and influence clot stability during administration of SD plasma and in two rare congenital disorders: FXIII deficiency and LPI.

The specific goals of the study were the following:

1) Assess the impact of SD plasma, congenital FXIII deficiency and LPI on thrombin generation and fibrinolysis
2) Combine various methods to aid in the understanding of a single target – clot stability
3) Identify factors that contribute to abnormal coagulation associated with LPI and design novel approaches of transfusion therapy accordingly

4 PATIENTS, MATERIALS, AND METHODS

4.1 Study subjects

In Study II, three patients with congenital FXIII-A deficiency who participated in the Novo Nordisk® mentorTM 2 trial (Carcao et al., 2017) were enrolled. Patient 1 was a 51-year-old woman (weight 81 kg, height 183 cm), patient 2 was a 58-year-old man (weight 103 kg, height 182 cm), and patient 3 was a 56-year-old woman (weight 102 kg, height 170 cm) with hypertension, intracranial hemorrhage (year 1988) and symptomatic epilepsy. Patient 3 had hydrochlorothiazide, valsartan and oxcarbazepine medications. Patients 2 and 3 were siblings. During the TM 2 trial, patients received rFXIII replacement therapy and were switched back to their previous prophylactic pdFXIII replacement when the trial ended. Doses of pdFXIII 12–15 IU/kg, pdFXIII 24–30 IU/kg or rFXIII 35 IU/kg were used to assess their impact on coagulation. Blood samples were collected from the patients before and 1 hour after FXIII concentrate administration. FXIII concentrate was administered once a month. FXIII trough levels for patient 1, 2 and 3 were 16 % (11–22 %), 24 % (16–32 %) and 15 % (10–20 %), respectively. Data are presented as median and range (n = 17).

Study III investigated 15 LPI patients (8 women), most of whom were renally compromised. The median age of these patients was 45 years (range of 23–60 years). 11 patients had regular low dose lysine substitution. Median lysine in plasma was 132 μmol/L (range 69–172 μmol/L), the normal range being 114–289 μmol/L, indicating low normal plasma lysine levels. The follow-up on LPI patients in Finland is centralized in Turku University Hospital, where plasma collection, whole blood coagulation, and the clinical analysis were performed.

Both clinical studies (II, III) with patient data followed the Institutional Guidelines of Good Clinical Practice. Informed consent was obtained in accordance with the Declaration of Helsinki and the hospitals` Institutional Review Boards and Ethics Committees approved these studies.

4.2 Plasma pools

Study I was an *in vitro* experimental design study, and no patient data were included. The study was conducted using several human plasma preparations. SD plasma products used in Study I were produced by Octapharma® (Lachen,

Switzerland). Brand names used were Octaplas® and Omniplasma® (Omniplasma® is collected and marketed only in The Netherlands). One unit of each SD plasma product was used in the study: Octaplas® blood group O and A, and Omniplasma® blood group A, B and AB. Sanquin plasma pool from healthy donors (n = 30) was used as the reference. The study was conducted using the Good Laboratory Practice principles.

Two batches of pooled reference plasma were obtained from two groups of healthy volunteers (n = 11 and n = 15) and used as a reference in Study II and III, respectively. Also, single donor FFP samples from healthy donors (n = 10) were collected and used as the reference in Study II and III.

4.3 Data collection

Data was gathered from patients during the trials and clinical characteristics were obtained from medical records (II, III). In Study I, data were collected from plasma preparations.

4.4 Blood samples

Plasma samples were collected by venipuncture and placed into vacuum tubes containing 3.2% sodium citrate (109 mM) following centrifugation (2500 x g, 10 minutes). Plasma was collected from the upper and middle third of the tubes, thereby avoiding contact with the buffy coat layer. Routine coagulation screening was performed, and the remaining plasma was stored at -80 ^{O}C. Citrated whole blood was used for PFA-100® and ROTEM® analysis with no centrifugation steps or manipulation.

4.5 Bleeding score (III)

Severity of bleeding tendency was scored using The International Society on Thrombosis and Haemostasis Scientific and Standardization Committee Bleeding Assessment Tool (ISTH/SSC-BAT) (Rodeghiero et al., 2010). Normal values for men were < 4 points and < 6 points for women (Elbatarny et al., 2014).

4.6 Metabolic Assays and Lipids (I, III)

In Study I, HDL, LDL, triglycerides, and VLDL were determined from the plasma preparations with centrifugation method. In Study III, total blood count, plasma alanine aminotransferase (ALT), plasma and urine creatinine (U-Crea), serum cystatin C, urinary amino acids, urine lysine (U-LYS), capillary blood gas analysis variables bicarbonate and base excess (BE), total plasma cholesterol, LDL, HDL and triglycerides were measured. Plasma creatinine was used to calculate the estimated glomerular filtration rate (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration CKD-Epi) equation (Levey et al., 2009). Urine lysine levels without creatinine correction were calculated by multiplying U-LYS with U-Crea.

4.7 Coagulation and fibrinolysis screening (I-III)

Basic coagulation screening was conducted with activated partial thromboplastin time (aPTT, Actin FSL®, Siemens Healthcare Diagnostics, Erlangen, Germany), thrombin time (TT; BC Thrombin reagent, Siemens Healthcare Diagnostics, Erlangen, Germany) and prothrombin time (PT; Nycotest PT® with the Owren buffer, Axis-Shield PoC As, Oslo, Norway). Automated coagulation analyses were done with the Sysmex CA-7000® analyzer (Siemens Healthcare Diagnostics, Erlangen, Germany).

The procoagulant factors FII, V, VII and X were determined in the Sysmex CA-7000 analyzer®, using one-stage clotting assays with Innovin®. FVIII, IX, XI and XII activities were evaluated on the Sysmex CA-7000® using Actin FSL®. Also, VWF activity (VWF:Ac) assay (INNOVANCE VWF Ac., Siemens Healthcare Diagnostics, Erlangen, Germany), FXIII:C activity (Berichrom chromogenic FXIII, Siemens Healthcare Diagnostics, Erlangen, Germany), fibrinogen (modified Clauss method, Multifibren U, Siemens Healthcare Diagnostics, Erlangen, Germany) and prothrombin fragments F1+2 (ELISA technique, Enzygnost monoclonal, Siemens Healthcare Diagnostics, Erlangen, Germany) were analyzed.

Natural anticoagulants antithrombin (a chromogenic assay, Berichrom Antithrombin III, Siemens Healthcare Diagnostics, Erlangen, Germany), free TFPI (Free TFPI, Asserachrom®, Diagnostica Stago S.A.S. Asnières sur Seine, France), total TFPI (Diagnostica Stago S.A.S. Asnières sur Seine, France), protein C (Siemens Healthcare Diagnostics, Erlangen, Germany), protein S (Diagnostica Stago S.A.S. Asnières sur Seine, France) and anticoagulant active single chain protein S (Dienava-Verdoold et al., 2012) were determined. Fibrinolysis regulators and the

markers, plasminogen and α2-antiplasmin (Diagnostica Stago S.A.S. Asnières sur Seine, France), TAFI activity (PEFA kit, Pentapharm, Basel, Switzerland), D-dimer (immunoturbidometric Tina-quant D-Dimer, Roche Diagnostics, Mannheim, Germany) and PAP (DRG Diagnostica, Marburg, Germany) were assessed.

4.8 Platelet function analyzer (PFA-100®) (III)

Platelet Function Analyzer (PFA-100®) (Siemens Healthcare Diagnostics, Erlangen, Germany) was used to investigate primary hemostasis. PFA-100® mimics the physiological high-shear force flow conditions in the cuvette with exposed collagen surface, and an epinephrine (EPI) or adenosine-5-diphosphate (ADP) spiked collagen cartridge will record the platelet plug formation as the closure times of collagen membrane cartridges. Reference ranges of 82–150 s (EPI) and 62–100 s (ADP) were used, and two parallel tests were run each time.

4.9 Thromboelastography (I) and Rotational Thromboelastometry (II-III)

TEG® (Hemoscope Corporation, Niles, IL, USA) was used in the first study to assess clot lysis time (CLT) in the plasma, defined as the time that passed between the maximal amplitude and 2 mm amplitude post maximal amplitude. TEG® was performed in the presence of 4 μM PL, 10 pM TF and 0.2 μg/mL tPA.

In ROTEM® (TEM International GmbH, Munich, Germany), EXTEM, INTEM, and FIBTEM tests were used. Tests were run up to 60 minutes to exclude late fibrinolysis. The recorded variables with their normal ranges are specified in Table 3. Both TEG® and ROTEM® were performed at 37^{O}C.

Table 3. *ROTEM® variables with their respective reference ranges (II-III).*

Variables		Reference Range
Clotting time (CT)	s	EXTEM: 38-79, INTEM: 100-240
Clot formation time (CFT)	s	EXTEM: 34-159, INTEM: 30-110
Maximum clot firmness (MCF)	mm	EXTEM and INTEM: 50-72, FIBTEM: 9-25
Maximum lysis (ML)	%	EXTEM and INTEM: 0-15

4.10 Thrombin generation with CAT® (I-III) and Gly-Pro-Arg-Pro (GPRP) (II)

A CAT® assay (Labscan, Thermo Fisher, Helsinki, Finland) using platelet poor plasma (PPP) was performed by the Thrombinoscope software (Thrombinoscope, Maastricht, The Netherlands), and the validated reagents (Diagnostica Stago, Asnières sur Seine Cedex, France) were used to measure TG *in vitro*. The reaction was triggered with 1 pM TF and 4 μM PL (PPP reagent low) in the absence of corn trypsin inhibitor. Lag time (time to initiation of thrombin formation, min), endogenous thrombin potential (ETP; the area under the curve; nM thrombin × min), peak (maximum thrombin concentration, nM), and α2M–thrombin complex (nM) were all measured. In Study I, APC and the anti-protein S antibody were added to the assay to determine APC-dependent and -independent protein S anticoagulant activity as described previously (Dienava-Verdoold et al., 2012).

Gly-Pro-Arg-Pro (GPRP) peptide was added to PPP before the CAT® assay was performed (II). GPRP inhibits fibrinogen polymerization by binding directly to the polymerization sites and reshaping the glutamine residues in both the α- and γ-chains of fibrinogen (Gallistl et al., 1995; Laudano et al., 1978). The final concentrations of GPRP were 0.2, 0.6 and 0.8 mM. The effect of dilution was controlled using the vehicle buffer.

4.11 Fibrin generation and fibrinolysis (I-III)

Viscoelastic POC methods TEG® and ROTEM® were used to examine clot stability and lysis in plasma (I) and whole blood (II-III). Fibrin formation and clot lysis were

assessed by the optical density (OD) of clotting plasma, triggered with TF and in the presence of tPA to induce fibrinolysis (TF, PL, tPA and $CaCl_2$ at final concentrations of 0.5 pmol/L, 4 μM, 50 ng/mL, and 15 mmol/L, respectively). OD_{max} was obtained directly from OD tracing measured at 405 nm. Clotting time (CT) and clot lysis time (CLT) were obtained from the first derivative of OD tracing. CT is the time elapsed from the addition of the reagent to maximum fibrin production. CLT is the time between maximum fibrin production and the maximum rate of clot lysis (III).

4.12 Statistical Analyses

In Study I, group comparisons were performed using a simple Student t-test, and in Study II and III, a Mann–Whitney test was used. In Study III, correlations were calculated based on the Spearman correlation coefficient; linear regression models were also used where pertinent. Prism 6.0d, 2013 by GraphPad Software, Inc. (La Jolla, California, USA), and IBM SPSS Statistics Version 21 (Armonk, New York, USA) were used for the calculations. Differences were considered significant at p-values ≤ 0.05.

5 RESULTS

5.1 SD plasma (I)

5.1.1 Enhanced thrombin generation

Two SD plasma products, Octaplas® and Omniplasma,® were used. Omniplasma® is manufactured using ligand gel technology, which shortens the SD treatment compared to Octaplas®. The TG in SD plasma was enhanced in CAT® compared to an untreated blood-group-specific single donor FFP and pooled reference plasma from Sanquin (n = 30). Of note, peak TG was 4-fold higher in SD plasma than in the reference plasma (Fig 7). Significant changes were also discovered in the lag time and ETP (Table 4). A higher amount of TF (5 pM) completely abolished differences in TG between plasma products.

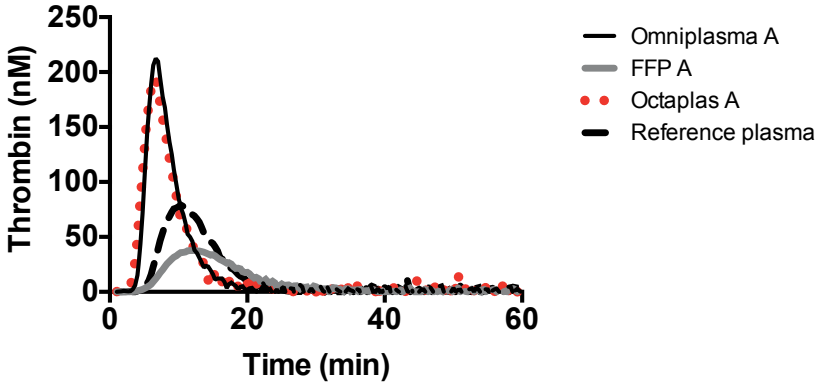

Figure 7. *Thrombin generation measured with CAT® in Octaplas® A and Omniplasma® A compared with a single donor FFP A and pooled reference plasma from Sanquin in 1 pM TF/ 4 μM PL. FFP = fresh frozen plasma; PL = phospholipids; TF = tissue factor*

Table 4. *Thrombin generation characteristics in SD plasma compared to pooled reference plasma from Sanquin in CAT®. Difference between SD plasmas and reference plasma calculated as fold with p-values to show statistical difference. Number of plasma batches used: Octaplas® n = 2, Omniplasma® n = 3.*

Plasma	**Lag time**		**ETP**		**Peak TG**	
	fold	*p*	fold	*p*	fold	*p*
Octaplas®	-1.5	*0.003*	2	*0.003*	4	*0.002*
Omniplasma®	-1.2	*0.009*	2	*0.0003*	4	*0.00003*

ETP = endogenous thrombin potential; TG = thrombin generation

Basic coagulation screen, procoagulant factors, antithrombin, and protein C were within the normal range in SD plasma samples, although the antithrombin levels in Octaplas® were moderately, but significantly, reduced compared to the reference plasma (Table 5). However, the amount of total TFPI was reduced to 62 % in Octaplas® compared to the reference plasma. The levels of total and free protein S antigens were significantly reduced in both Octaplas® (67 % and 71 %, respectively) and Omniplasma® (87 % and 86 %, respectively) compared with reference plasma. Furthermore, the amount of active single chain protein S was only 12–14 % in both Omniplasma® and Octaplas® (Table 5). In the following investigations, the addition of APC reduced peak TG in Octaplas® ($p = 0.02$) and Omniplasma® ($p = 0.0002$), but not in the reference plasma, thereby supporting diminished APC-cofactor activity in the SD plasma. In futher experiments, the addition of the protein S inhibitory monoclonal antibody CLB-PS 13 enhanced the peak TG in reference plasma, but not in the SD plasma samples ($p < 0.0005$), indicating the loss of APC-independent anticoagulant activity of protein S during the SD process.

Since LDL and VLDL are recognized to carry TFPI, and protein S was identified in the proteome of VLDL, we measured the lipid content of SD plasma samples. The levels of HDL remained unaltered, but both LDL and VLDL were significantly reduced in Octaplas® and Omniplasma® due to the SD treatment (Table 5) (Pitkänen et al. 2018. Unpublished data).

Table 5. *Levels of the inhibitors of thrombin generation (TG), fibrinolysis markers and lipids in Octaplas®, Omniplasma® and the reference plasma (ref. plasma) obtained from healthy volunteers. Data are presented as mean ± standard deviation. Number of plasma batches used, please see Table 4.*

Marker		Normal range	Ref. plasma	Octa-plasma®	Omni-plasma®
TG inhibitors					
AT	%	80-120	97±3	83±3*	90±3
PC	%	75-125	111±13	96±0	100±2
PS total ag	%	60-140	98±6	66±2**	86±5
PS free ag	%	60-140	95±4	68±3**	82±1*
PS intact ag	% of total ag		65±0	14±0***	12.5±0.5***
TFPI total ag	ng/mL	30.4-72.9	56.5±2.8	35.1±1.7*	55.5±0.4
TFPI free ag	ng/mL	5.7-16.6	8.7±2.0	8.9±0.4	8.9±0.6
Fibrinolysis markers					
Plasminogen	%	80-120	110±11	91±0	88±1
α2-AP	%	80-120	114±20	23±3*	43±4*
Lipids♣					
HDL	mmol/L	w > 1.2 m > 1.0	1.3±0.1	1.5±0.01	1.6±0.01
LDL	mmol/L	< 3	2.3±0.2	0.2±0.01***	0.2±0.02***
VLDL	mmol/L		0.50±0.06	0.003±0***	0.005±0**

α2-AP = α2-antiplasmin; AT = antithrombin; HDL = high-density lipoprotein; LDL = low-density lipoprotein; PC = protein C; PS = protein S; ag = antigen; TFPI = tissue factor pathway inhibitor; VLDL = very low-density lipoprotein; w = women; m = men ♣ = Unpublished data by Pitkänen et al 2018
*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$

5.1.2 Increased fibrinolysis

In SD plasma, plasminogen activity was normal compared to reference plasma, but α2-AP levels were decreased (Table 5). TEG® CLT was 7 ± 1 min shorter in SD plasma

than in the reference plasma ($p < 0.0005$) indicating increased fibrinolysis. CLT could be normalized with TXA at a final concentration of 2.5 μg/mL.

To summarize, *in vitro* TG was enhanced in SD plasma and markedly reduced levels of single chain protein S were discovered. Further, SD treatment reduced the levels of VLDL and intact protein S antigen levels, and both the APC-cofactor and APC-independent anticoagulant activity of protein S were diminished. In addition, fibrinolysis was increased, and decreased levels of α2-AP were detected. Fibrinolysis was corrected with TXA.

5.2 Congenital FXIII deficiency (II)

5.2.1 Thrombin generation

In vitro TG measured with CAT® was enhanced at the trough FXIII level, showing a 1.6 to 1.8-fold increase compared with the pooled reference plasma. Patients 2 and 3 reached normal levels of *in vitro* TG as a response to FXIII replacement therapy, while the peak TG levels of Patient 1 remained elevated compared to the reference plasma (Fig 8A). Furthermore, Patient 1 showed a 2.8-fold increased *in vivo* TG ($p < 0.005$), assessed with prothrombin fragments F1+2 both at trough and recovery, while Patient 2 had a 1.6-fold increase ($p < 0.05$) and Patient 3 remained at the high normal range compared to the healthy controls (Fig 8B). The dosage or the regimen of FXIII replacement therapy did not impact TG *in vitro* or *in vivo*. Basic coagulation screen and procoagulant factor assays were used to exclude any other coagulation factor abnormalities. Normal prothrombin, AT and α2M–thrombin complex levels suggest that enhanced TG associates with overactive thrombin conversion.

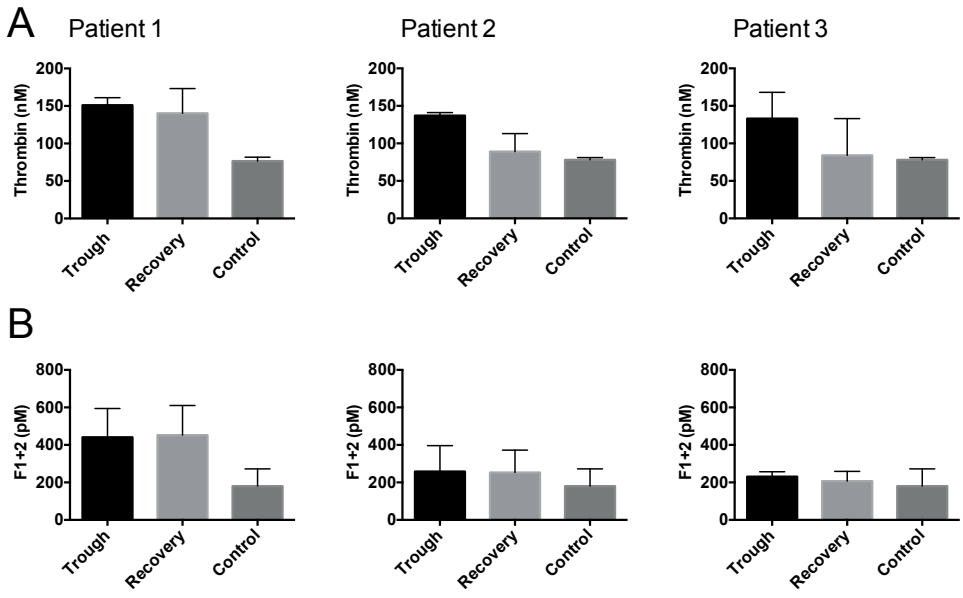

Figure 8. *In vitro (A) and in vivo (B) thrombin generation (TG) measured from all three FXIII deficiency patients at trough (n = 3) and 1-hour recovery (n = 3) compared with healthy controls. In vitro TG presented with peak TG in CAT® and in vivo TG analyzed with circulating prothrombin fragments F1+2 (reference range 69–229 pM).*

In further experiments, we inhibited fibrin polymerization *in vitro* by adding GPRP to the reference plasma and patient FXIII recovery plasma in CAT®. GPRP reinforced TG in a dose-dependent manner (Fig 9), while fibrinogen clotting activity was simultaneously impaired as determined by the turbidity changes using the Clauss method.

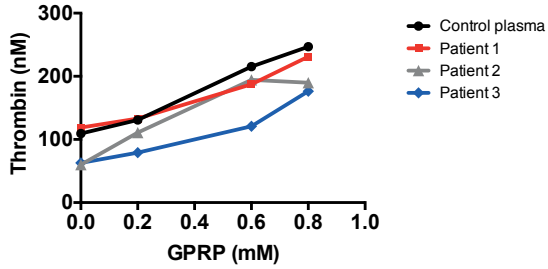

Figure 9. *GPRP dose-response curve plotted against CAT® peak thrombin generation (nM). Reference plasma and patient FXIII 1-hour recovery plasma with final concentrations of 0.2 mM, 0.6 mM, and 0.8 mM GPRP being used. Peak thrombin generation was enhanced in a dose-dependent manner, as GPRP concentration was increased.*

5.2.2 Fibrin clot formation and fibrinolysis

Patients 1 and 2 with FXIII deficiency had normal fibrinogen levels, whereas Patient 3 had mildly elevated levels (Fig 10A). As expected, fibrinogen levels remained unaffected by FXIII replacement, and functional fibrin clot formation, evaluated with FIBTEM maximum clot firmness (MCF), increased in a dose-dependent manner (Fig 10B). Despite the normal FXIII levels at recovery, only Patient 3 with elevated fibrinogen levels reached a normal range in FIBTEM MCF. Both pdFXIII (24–30 IU/kg) and rFXIII (35 IU/kg) could normalize the FXIII levels, while the low dose of pdFXIII (12–15 IU/kg) resulted in half the response (Fig 10C). D-dimer and maximum lysis (ML) in EXTEM and INTEM were within normal ranges at trough and recovery, excluding major fibrinolysis.

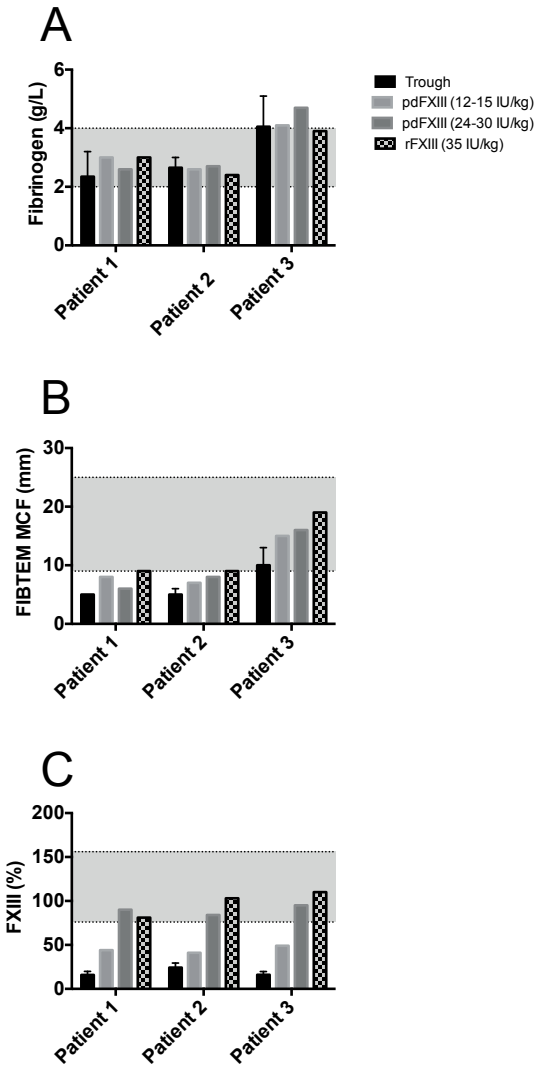

Figure 10. *Three patients with congenital FXIII deficiency. Fibrinogen (A), FIBTEM MCF (B), and FXIII levels (C) at trough and 1-hour recovery after each regimen of FXIII replacement. Normal range is shaded in grey. Trough data presented as median and range (n = 4). Recovery measured once after each regimen. MCF = maximum clot firmness*

Briefly, in FXIII deficiency, both *in vivo* and *in vitro* TG were enhanced at trough. *In vitro* TG was partially corrected by FXIII replacement, but *in vivo* TG remained increased despite the replacement therapy. Low levels of fibrinogen and FXIII resulted in poor fibrin generation and clot formation detected by FIBTEM MCF. Corrections of the FXIII levels improved clot formation, while only mildly elevated, not normal, fibrinogen levels produced clots with normal firmness. Congenital FXIII

deficiency patients were not susceptible to lysis even at trough levels when assessed with D-dimer and ROTEM® ML.

5.3 LPI (III)

5.3.1 Altered thrombin generation

In vivo TG was markedly elevated in LPI as seen by increased levels of circulating prothrombin fragments F1+2. It exceeded 1200 pM (ranging from 961 to over 1200 pM), whereas in healthy individuals the median F1+2 was 178 pM (range 91–272 pM). In contrast, *in vitro* TG in CAT® decreased as displayed by lower ETP (median 754 nM x min; range 444–1266 nM x min) than in healthy controls (median 1165 nM x min; range 542–1688 nM x min) ($p = 0.02$). A basic coagulation screen was performed to exclude any coagulation factor deficiencies, and the screen did not show any. Furthermore, TAFI activity did not differ from the controls ($p > 0.05$).

5.3.2 Decreased clot formation

4 LPI patients had fibrinogen levels below the normal range, and 11 patients had FXIII values below normal range, leading to impaired clot formation as assessed with OD derivatives OD_{max} ($p < 0.03$) and CT ($p < 0.001$) (Fig 11). All 4 patients with low fibrinogen levels also had low FXIII levels. To add, FIBTEM MCF was diminished in accordance with the reduced levels of fibrinogen, as 7 patients had levels below the normal range (median 9 mm; range 3–20 mm).

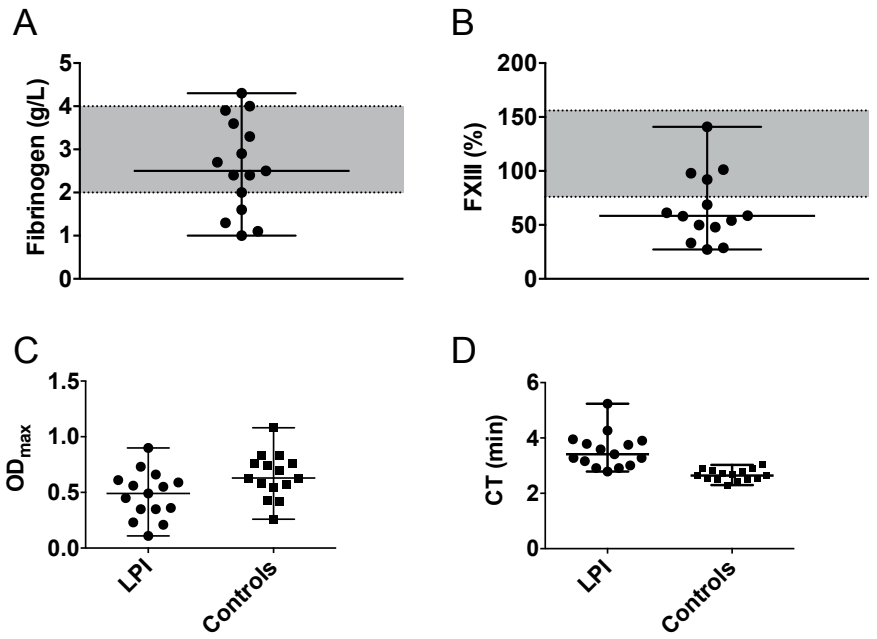

Figure 11. *Markers of clot formation in LPI. Fibrinogen (A) and FXIII (B), reference range is shaded in grey. Optical density maximum (OD_{max}) (C) and clotting time (CT) (D) were compared to healthy individuals. The lines represent median and range.*

5.3.3 Fibrinolysis

Median plasminogen was elevated, while α2-AP levels were lower leading to a higher plasminogen/α2-AP ratio than in the healthy controls. An imbalance between major fibrinolysis regulators was reflected as a shortened OD derivative CLT in LPI when compared to the healthy controls (Table 6) (III).

Table 6. *Major fibrinolysis regulators and clot lysis time (CLT) as derived from the optical density experiment in LPI and healthy controls. Data are presented as a median and range.*

Fibrinolysis regulators and markers		Controls	LPI patients
Plasminogen	%	99 (92-111)	120 (91-155) *
α2-antiplasmin	%	101 (95-113)	88 (50-105) **
Pls/α2-AP ratio		0.96 (0.82-1.16)	1.50 (1.10-2.02) **
CLT	min	42 (31-91)	30 (24-49) **

Pls/α2-AP ratio = Plasminogen/α2-antiplasmin ratio; *$p = 0.01$, **$p < 0.001$

D-dimer levels were extremely high in LPI (median 32 mg/L; range 12–109 mg/L), and PAP exceeded 2000 μg/L and the calibration curve range, while in the healthy controls median PAP was 638 μg/L (range 430–862 μg/L). EXTEM and INTEM ML were within the normal range in LPI despite high levels of FDPs.

To summarize, in LPI, *in vivo* TG was notably magnified, but *in vitro* TG was significantly reduced. Low levels of fibrinogen and FXIII resulted in decreased fibrin generation and clot formation determined with OD_{max} and FIBTEM MCF. The LPI patients also presented with enhanced fibrinolysis manifested by shortened CLT and extreme D-dimer and PAP levels. An elevated plasminogen/α2-AP ratio was discovered.

5.4 Bleeding disorder and organ manifestations in LPI (III)

5.4.1 Bleeding score

The median ISTH/SSC-BAT bleeding score (BS) was 4 points (range 0-9 points). Only one spontaneous (gastrointestinal) bleed was reported. 9 patients presented with normal BS, but only one man was subjected to minor surgery without bleeds, whereas 7 patients reported bleeding complications due to invasive procedures. 2 women had normal labor and puerparium, while 2 experienced severe postpartum hemorrhage. Based on the BS, LPI patients are prone to mucocutaneous bleeds as hemorrhage from minor wounds, the oral cavity, after tooth/teeth extraction, or after surgery or major trauma. Menorrhagia and post-partum bleeds were also reported.

5.4.2 Impaired primary hemostasis

Impaired primary hemostasis was reflected in LPI by mild thrombocytopenia and poor primary hemostasis in PFA-100®. Only one patient had a platelet count under 100 E^9/L (Fig 12), however. VWF activity was normal.

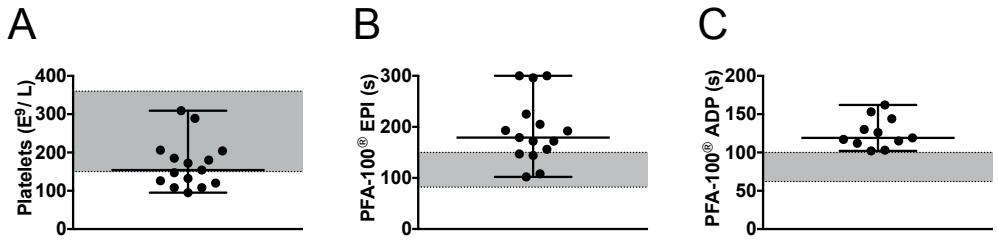

Figure 12. *Platelets (A), PFA-100® EPI (B) and ADP (C) in LPI. The patients presented with mild thrombocytopenia (A) and impaired primary hemostasis in PFA-100® (B, C). PFA-100® ADP was obtained from 11 patients. Lines represent median and range, and the normal range is shaded in grey. PFA-100® = platelet function analyzer; EPI = epinephrine; ADP = adenosine-5-diphosphate*

5.4.3 Lipid profile and liver function

In our study, 10 patients had cholesterol medication, and 4 attained normal cholesterol values, while 6 remained hypercholesterolemic. One patient presented with normal cholesterol values without medication. Total cholesterol was elevated in 10 patients. LDL was measured in 12 patients and 4 had values below <3 mM. Further, 3 patients had normal triglycerides and 10 patients had combined hyperlipidemia with simultaneously elevated total cholesterol and triglycerides. Also, 5 patients had normal HDL, while the rest had levels under the normal range (Table 7). 3 male patients had mildly elevated ALT and all patients had normal PT, excluding major liver dysfunction.

Table 7. *Lipid values among LPI patients. LDL was obtained from 12 patients. Data are presented as a median and range.*

Lipids		**Normal range**	**LPI patients**
Total cholesterol	mM	< 5	5.8 (3.8-8.6)
LDL	mM	< 3	3.5 (2.0-4.4)
HDL	mM	w> 1.2 / m> 1.0	w: 1.2 (1.1-1.4) / m: 1.1 (0.5-1.3)
Triglycerides	mM	< 2	2.6 (0.9-7.1)

HDL = high-density lipoprotein; LDL = low-density lipoprotein; w = women; m = men

5.4.4 Correlation of renal insufficiency and fibrinolysis

The LPI patients had moderate renal insufficiency and eGFR and serum cystatin C were affected. As a sign of systemic acidosis, the base excess was negative and bicarbonate was low. Lysine leaked into urine, and urine lysine levels were increased (Table 8).

Table 8. *Renal and metabolic markers in LPI patients. 12 patients had low estimated glomerular filtration rate (eGFR), while cystatin C was elevated in 10 patients. Capillary blood gas analysis was obtained from 13 patients; 10/13 patients had low bicarbonate levels and base excess levels. Data are presented as a median and range.*

Renal and Metabolic Markers		Normal Range	LPI patients
eGFR	mL/min/m^2	*	44 (16-105)
Cystatin C (serum)	mg/L	0.6-1.1	1.4 (0.6-2.5)
Lysine (urine)	μmol/mmol Crea	2-63	406 (112-1076)
Bicarbonate (whole blood)	mM	22-26	21 (13-23)
Base excess (whole blood)	mM	-2.5 to +2.5	-4.2 (-10.5 to -1.3)

* Normal range for eGRF: age 18-39 years > 89; 40-49 years > 83; 50-59 years > 77; 60-69 years > 69

eGFR and cystatin C correlated inversely with the lysine levels in urine (Fig 13). Remarkably, the loss of renal function correlated with diminished fibrin formation and enhanced fibrinolytic capacity. eGFR, cystatin C, bicarbonate, and base excess correlated with decreased OD_{max} and α2-AP and elevated D-dimer (Fig 14).

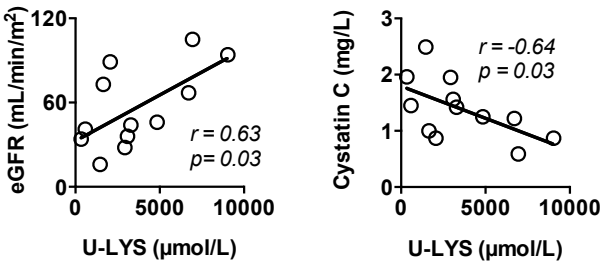

Figure 13. *Spearman correlations of renal function markers estimated glomerular filtration rate (eGFR) and cystatin C with urine lysine (U-LYS) levels.*

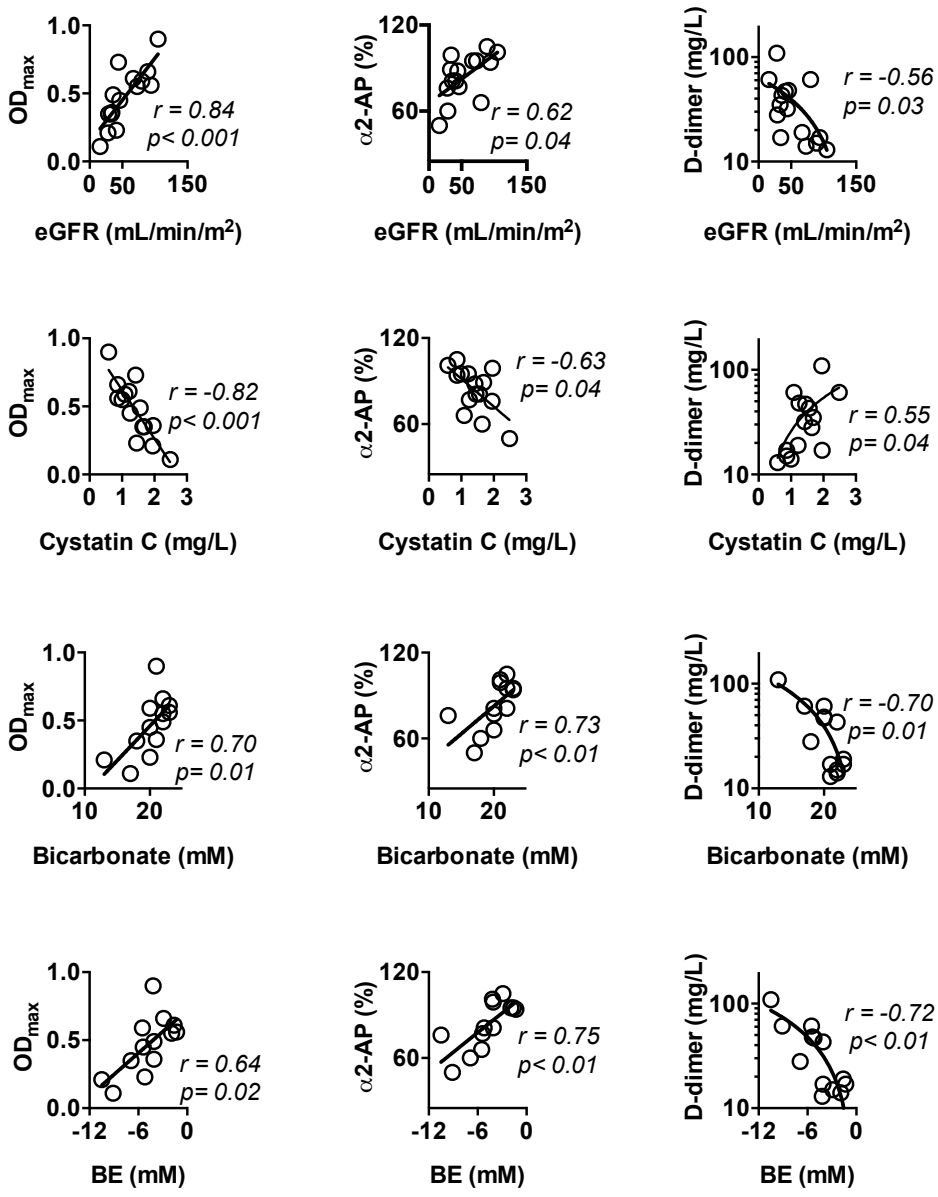

Figure 14. *Spearman correlations of diminished fibrin formation (OD_{max}) and markers of enhanced fibrinolysis (α2-AP and D-dimer) with renal function (eGFR and cystatin C) and variables' indicating systemic acidosis (bicarbonate and BE). D-dimer is presented in logarithmic scale. α2-AP = α2-antiplasmin; BE = base excess; eGFR = estimated glomerular filtration rate; OD_{max} = optical density maximum.*

In summary, spontaneous bleeds are rare in LPI. However, invasive procedures render LPI patients susceptible to bleeding complications. Hyperlipidemia does not seem to increase thrombosis risk for these patients. Primary hemostasis was decreased despite normal VWF activity levels, and markers of fibrinolysis were magnified. Elevated urine lysine correlated inversely with the level of renal insufficiency, and markers of fibrinolysis correlated with the loss of renal function and increased acidosis.

6 DISCUSSION

In clinical emergency settings, we do not try to decipher complicated coagulation systems. The focus of attention is a bleeding patient who is in critical condition, while the treatment options and monitoring tools are scarce. MT protocols, goal-directed guidelines, and viscoelastic coagulation POC instruments are crucial in this milieu. However, in elective situations perioperative planning leading to personalized treatment is possible and is based on complicated feedback loops that might lead to bleeds, adequate hemostasis, or thrombosis. Retrospective analysis of the emergency management of hemostasis is thus valuable for future events, and therein these elective procedures provide critical opportunities for re-evaluations and development of improved approaches.

This thesis sought to identify the factors related to TG and clot stability that can be modified by transfusion or concentrate therapy to maintain sufficient hemostasis and prevent bleeding or thrombosis. These pathways were monitored using both traditional and functional coagulation screening methods under stable non-bleeding conditions without factor and blood cell consumption. SD plasma showed enhanced TG and fibrinolysis, which could be at least partially caused by decreased single chain protein S and α2-antiplasmin activity. Fibrinolysis was effectively corrected by the administration of TXA *in vitro* (I). Low, but hemostatically sufficient trough levels of FXIII (Menegatti et al., 2017) induced accelerated prothrombin conversion leading to increased TG, possibly based on decreased fibrin AT I–like activity (II).

In addition, this thesis aimed at determining the pathophysiology behind the bleeding tendency in LPI patients with various degrees of renal impairment and metabolic disorder. We discovered impaired primary hemostasis, altered TG, decreased fibrin formation and enhanced fibrinolysis related not only to increased plasminogen/α2-AP ratio, but with metabolic acidosis and renal insufficiency (III).

6.1 Thrombin generation *in vivo* and *in vitro*

SD treatment of plasma strongly reduced the level of single chain protein S activity, which could be a contributing factor to enhanced *in vitro* TG (I). At present, the underlying mechanisms causing low levels of single chain protein S in SD plasma remain unknown. Possible explanations include altered lipid composition in SD

plasma due to SD treatment (discussed at length in page 60), previously detected protease with protein S clevage capacity released from platelets during SD treatment (Brinkman et al., 2005), and conformational changes in protein structure caused by denaturation during SD treatment (Mast et al., 1999).

The potency of TG in plasma (*in vitro* TG) is not equal to the extent of ongoing thrombin formation in the body (*in vivo* TG). When a thrombus is forming, both prothrombin fragments F1+2 and the TAT complex appear in the circulation (Al Dieri et al., 2012). Patients with FXIII deficiency showed increased *in vivo* and *in vitro* TG at trough. Normal levels of prothrombin, AT and α2M–thrombin complex propose that enhanced TG may associate with overactive thrombin conversion. Two of the three patients normalized TG at recovery *in vitro*, but *in vivo*, TG remained enhanced (II). Increased *in vivo* TG is a sign of an ongoing pathological process (van der Poll et al., 1990), whereas elevated or decreased *in vitro* TG suggests abnormal functioning of the coagulation process. Altered *in vitro* TG reflects an increased risk of thrombosis or bleeding. Furthermore, *in vivo* TG strongly depends on the amount of TF and TM adjacent to the vascular endothelium (Al Dieri et al., 2012). *In vitro* TG measures the hemostatic balance of clotting factors and inhibitors, without the impact of pro- and anticoagulant drivers that are produced by the vascular endothelial cells (Tripodi, 2016). Intriguingly, both markedly increased *in vivo* TG, but significantly reduced *in vitro* TG was present in LPI (III). This discovery might refer to the consumption coagulopathy proposed by Kayanoki et al (Kayanoki et al., 1999) and supported by our findings of elevated F1+2, D-dimer and PAP. However, mild thrombocytopenia, normal PT, aPTT, and VWF activity suggest otherwise (III).

6.2 Interplay between thrombin generation and fibrin formation

The structure of fibrin depends on various factors, including pH, ionic strength and concentrations of calcium, fibrinogen, and thrombin. Thrombin plays an integral role in determining fibrin strand thickness and the density of a fibrin clot, as well as in activating the platelets. Low concentrations of thrombin produce loose clots with thick fibrin strands being susceptible to fibrinolysis, whereas high thrombin levels result in densely packed clots and thin strands that are more resistant to lysis (Wolberg et al., 2008). TAFI inhibits fibrinolysis by down-regulating the cofactor functions of partially degraded fibrin. In the presence of TM, thrombin stimulates the activation of both TAFI and protein C, whereas APC suppresses TAFI activation by diminishing TG. Low TM concentrations activate TAFI and inhibit fibrinolysis. In contrast, high TM concentrations lead to the activation of protein C and increased fibrinolysis (Mosnier et al., 2001).

However, fibrinogen can also regulate thrombin. Fibrinogen can stimulate the conversion of prothrombin to thrombin. Further, the binding of thrombin to plasma fibrin(ogen) protects thrombin from its inhibitors (Kremers et al., 2014, 2015). The effects of basal fibrinogen levels on clot formation and AT I–like activity of forming fibrin as a physiological down-regulator of TG (Mosesson, 2007) could present a plausible explanation to our findings in congenital FXIII deficiency. Patient 3 with elevated fibrinogen levels had the most effective clot formation in FIBTEM and the lowest peak TG *in vitro*, whereas Patient 1 had low normal fibrinogen levels, but weak clot formation in FIBTEM and high peak TG *in vitro*. Further, the inhibition of fibrin polymerization by GPRP led to increased TG *in vitro* (II). Finally, in LPI as an example, the net effect of clot formation was defined by altered TG, both *in vivo* and *in vitro*, low to normal basal fibrinogen levels, and decreased FXIII activity, resulting in delayed clot formation and stability when assessed with OD derivatives and FIBTEM (III).

6.3 Fibrinolysis

6.3.1 Inherited secondary fibrinolysis in LPI

Hyperfibrinolysis can be divided into primary and secondary forms, and it can be either inherited or acquired. In primary hyperfibrinolysis, an absolute quantitative or qualitative deficiency of antifibrinolytic proteins is present, whereas in secondary hyperfibrinolysis, either an abnormal consumption of structurally normal fibrinolytic enzymes or increased susceptibility of fibrin to lysis is detected (Kolev et al., 2016). All LPI patients share inherited recessive autosomal biallelic mutations (Borsani et al., 1999). Also, in our study the balance between plasminogen and α2-AP was altered, which could lead to enhanced fibrinolytic activity, as evidenced by extreme D-dimer and PAP levels and decreased CLT (III). It seems plausible, therefore, that LPI patients have inherited secondary hyperfibrinolysis, wherein hyperfunction is provoked in response to overt abnormal systemic blood clotting. In LPI, a common genetic defect is an underlying cause, but spontaneous bleeds are rare in contrast to homozygous α2-AP deficiency (Kluft et al., 1982). Liver cirrhosis provides a resembling clinical condition, characterized by increased clotting activity and secondary hyperfibrinolysis in which the hemostatic system has been rebalanced. Like LPI patients, patients with chronic liver cirrhosis do not have a tendency to bleed spontaneously. Still, the risk of bleeds must be assessed before any invasive procedures (Blasi, 2015).

6.3.2 The role of lysine residues in fibrin formation and fibrinolysis

Lysine residues play an interesting role in both fibrin formation and fibrinolysis. For example, FXIIIa crosslinks fibrin polymers into fibrin fibers by forming covalent bonds between the α- and γ-chain lysine and glutamine residues (Kanaide et al., 1975; Lorand, 1972). In addition, fibrinolysis is stimulated to increase 10^2–10^3-fold when tPA is activated with plasminogen on a fibrin surface. Both tPA and plasminogen have fibrin binding sites on their kringle domains, which often contain lysine binding sites that bind to internal and C-terminal lysine residues. C-terminal lysine residues generated by plasmin on fibrin are especially important as a positive feedback mechanism for the stimulation of fibrinolysis (Longstaff et al., 2015). During fibrin degradation plasmin proteolysis generates more C-terminal lysines providing new binding sites for plasmin and plasminogen (Silva et al., 2012). TAFI cleaves the same C-terminal lysines thereby inhibiting fibrinolysis, whereas α2-AP binds to plasminogen lysine binding sites, where fibrin is also non-covalently bound (Longstaff et al., 2015; Lee et al., 2004). Soluble lysine analogues, such as TXA, can be used to inhibit hyperfibrinolysis by binding to plasminogen and plasmin kringle domains to block interaction with fibrin (Kolev et al., 2016).

6.3.3 Hyperfibrinolysis and tranexamic acid

In SD plasma, the fibrinolysis could be corrected with TXA *in vitro* (I). At the target circulating concentration (200 μmol/L), TXA inhibits tPA activation of plasminogen on fibrin, but higher concentrations are needed to block plasmin. However, under certain specific conditions, TXA can be profibrinolytic, hence causing elevated mortality when given more than 3 hours after trauma. TXA can induce conformational changes rendering plasminogen more susceptible to uPA activation in the presence or absence of fibrin (Silva et al., 2012; Sinniger et al., 1999). Under poor fibrin formation levels, TXA also protects plasmin from α2-AP activity (Longstaff et al., 1991; Thelwell et al., 2007) and facilitates the diffusion of plasmin through a fibrin clot as a result of the diminished binding to C-terminal lysines (Kovács et al., 2014). The role of TXA, a lysine analogue, in LPI is unknown. Whether the administration of a lysine analogue in LPI would be beneficial or lead to exponential lysis has not yet been explored.

6.3.4 Fibrinolysis in congenital FXIII deficiency

A previous study shows that approximately 50 % of normal plasma FXIII levels provide sufficient protection against premature fibrinolysis in platelet poor plasma (Mutch et al., 2010). It is debated whether the antifibrinolytic function of FXIII is independent of fibrin–fibrin crosslinking. Under flow conditions in plasma, the

antifibrinolytic effect seems to be expressed through the ability of FXIII to crosslink α2-AP to fibrin (Fraser et al., 2011). In contrast, earlier reports with purified proteins suggest that fibrin-fibrin crosslinking inhibits fibrinolysis (Francis et al., 1988; Siebelist et al., 1994). According to a more recent study, susceptibility of tPA-catalysed fibrinolysis may be explained by reduced crosslinking and altered clot structure in the absence of FXIII (Hethershaw et al., 2014). In terms of mechanical clot stability, FXIII levels as low as 5 % may offer protection against serious hemorrhagic events (Nugent et al., 2015), but minimal FXIII activity level of 15 % is recommended in order to avoid major spontaneous bleeds (Menagetti et al., 2017). All three patients in our study had trough FXIII levels over 15 %. Their D-dimer levels were normal and ROTEM® could not show fibrinolysis (II). Administration of 50 IU/kg rFXIII concentrate has been shown to decrease fibrinolysis in TEG® (Lovejoy et al., 2006). In our study with smaller dose of rFXIII concentrate, we did not detect changes in fibrinolytic activity as a response to FXIII concentrate, although an increased clot firmness was discovered with ROTEM® (II).

6.4 Plasmin and hyperfibrinolysis in renal disease

Renal insufficiency in general is associated with impaired primary hemostasis (Islam et al., 2010; Ranghino et al., 2014), hypercoagulability and altered fibrinolysis (Adams et al., 2008; Mezzano et al., 1996). However, in LPI (III) the magnitude of both hypercoagulation and hyperfibrinolysis is more extravagant than in previous studies exploring coagulation in renal insufficiency (Lindner et al., 2014; Mezzano et al., 2001).

Renal impairment with tubular and, more rarely, glomerular lesions is a serious complication of LPI (Nicolas et al., 2016; Tanner et al., 2007). In our work here, moderate renal insufficiency with systemic acidosis and elevated renal markers was present and increased levels of urine lysine correlated inversely with the severity of renal impairment (III).

We discovered an elevated plasminogen/α2-AP ratio in LPI (III), which might induce fibrinolytic drive and elevated plasmin levels, known to be detrimental to kidneys in experimental animal settings (Edgtton et al., 2004). Besides the liver, plasminogen is expressed by renal medulla and cortex, possibly leading to high local concentrations of plasmin followed by renal damage (Zhang et al., 2002). Both disrupted fibrin generation and increased fibrinolysis correlated strongly with the extent of renal insufficiency and systemic acidosis in our study, thereby revealing a potential specific link between renal impairment and bleeding diasthesis in LPI (III).

In our study, the LPI patients presented with clearly impaired primary hemostasis observed in the PFA-100® analysis under a high-shear rate blood flow (III). In addition to the prior role of arginine and NO (Mannucci et al., 2005; Radomski et al., 1990), other factors should be considered as well. Platelet adhesion to the vessel wall depends on the VWF and GpPIb axis. Since VWF activity was normal, it is possible that GpIb could be proteolytically altered by plasmin, resulting in impaired platelet adhesion (Adelman et al., 1985). Further, platelet function is decreased in chronic renal failure due to factors such as disturbed α-granule content and reduced platelet adhesion and aggregation (Lutz et al., 2014). For LPI patients, the combination of altered plasmin levels and kidney impairment may have a detrimental impact on primary hemostasis.

6.5 Impact of lipid profile on coagulation

In SD plasma, the levels of LDL and VLDL were significantly decreased compared to the reference plasma (Pitkänen et al 2018. Unpublished data), as previously reported by others (Hellstern et al., 2011b). Protein S has been identified in the proteome of VLDL (Dashty et al., 2014) and indeed, we discovered reduced levels of protein S free antigen in both Octaplas® and Omniplasma®. Both LDL and VLDL are identified as TFPI carriers (Wesselschmidt et al., 1992), and total TFPI level was clearly reduced in Octaplas® with a relatively long SD exposure. However, TFPI levels did not differ from reference plasma in Omniplasma® with a reduced SD treatment time. These findings suggest a link between lipid content of SD-treated plasma products and coagulation activity. Moreover, lipoprotein(a) is known to inhibit plasminogen activation (Hancock et al., 2003). Although we did not measure lipoprotein(a) in Study I, delipitation caused by SD treatment is likely to reduce lipoprotein(a) levels, thus potentially increasing fibrinolysis further when combined with reduced α2-AP levels.

In a previous study, hypertriglyceridemia has been shown to inhibit fibrinolysis via the PAI-1 mediated pathway (Lowe et al., 1982). In contrast to these findings, LPI patients presented with hyperlipoproteinemia, hypertriglyceridemia and enhanced fibrinolysis in our study (III). Cationic amino-acid transporter protein y^+LAT-1 is not expressed in hepatic tissue and major liver disease has not been detected related to LPI, although hepatosplenomegalia and early-onset hepatic fibrosis have been reported (Shinawi et al., 2011). The hepatic clearance of both lipids and activated coagulation factors is mediated by the LRP1 receptor. Markers of coagulation and fibrinolysis, such as the prothrombin fragments F1+2, TAT, D-dimer and PAP, are

breakdown products and protease-inhibitor complexes with a short half-life (Lisman et al., 2007). Hepatic clearance of these molecules might be prolonged in LPI due to LRP1 receptor exhaustion that is induced by combined hyperlipidemia and early-onset fibrosis. In summary, extreme levels of the prothrombin fragments F1+2, D-dimer and PAP in LPI could, at least partially, reflect impaired hepatic clearance, but not consumption coagulopathy as previously suggested (Kayanoki et al., 1999).

6.6 Perioperative care in rare bleeding disorders – LPI as an example

LPI patients are prone to bleeds that are provoked by invasive procedures and deliveries (Tanner et al., 2006); therefore, their coagulation status should be carefully assessed perioperatively. Based on our study, traditional coagulation screening with aPTT, TT, and PT will not assist in understanding the coagulopathy in LPI. Clinical application of a TG assay could be a better marker of adequate hemostasis in LPI, if and when it is commercially available (Kintigh et al., 2018). Thrombocytopenia was mild, but even with normal platelet counts and VWF activity, PFA-100® closure times were prolonged. We recommend testing primary hemostasis with PFA-100®, and platelets should be given preoperatively, especially in high bleeding risk procedures. Fibrinogen and FXIII concentrations should be measured and replacement administered, if below the normal range. High levels of fibrinolysis markers are distinct for LPI, and although possibly partially due to impaired hepatic clearance, they are not suitable for thrombosis diagnostics in LPI. The utility and safety of TXA are still issues that remain to be studied in LPI.

ROTEM® was used to evaluate the bleeding tendency in LPI. In our study, ROTEM® was able to identify patients with low fibrinogen and FXIII levels, and it can thus be recommended as a repetitive viscoelastic POC method in operative settings and emergencies. Overall, ROTEM® can be a valuable tool when treating the patients with certain rare bleeding disorders, when the diagnosis is known in advance under a stable basal condition. However, ROTEM® is not a tool for differential diagnostics in rare bleeding disorders.

6.7 Strengths and Limitations of the study

Information gained from the *in vitro* coagulation tests is limited due to the absence of input caused by surrounding cellular surfaces and vascular structures. In contrast,

the *in vivo* tests including all physiological and pathophysiological factors impacting the coagulation system can obscure the interpretations and diagnostics (Mann et al., 2009). The strength of this study, however, was in combining both the *in vivo* and *in vitro* methods to assess the coagulation system from a broader perspective. We included several unique plasma preparations and patient profiles and phenotypes to achieve this goal.

Study I was experimental, and although our results were indeed compelling, further investigation is needed to fully evaluate the clinical implications of our findings. High levels of TF abolished the differences in TG in CAT® between plasma products *in vitro* (I), indicating that the methods and reagents being used can override the actual real-life coagulation potential.

Due to the lack of availability of local laboratory services, the second step of centrifugation before plasma conservation could not be conducted in Study III. The absence of a second centrifugation reduces the reliability of the results for most coagulation tests, since the remaining platelets provide anionic PL surfaces upon thawing/frosting cycles (Hemker et al., 1983). We also recognize the lack of a control group with kidney impairment in Study III, as LPI patients were only compared with healthy individuals. Renal insufficiency is caused by a wide variety of different underlying conditions with highly specific alterations in the coagulation and fibrinolytic system (Madhusudhan et al., 2016), making the choice of the potential control group difficult. Intriguingly, hantaviruses cause LPI-resembling acute condition with renal insufficiency and relatively similar alterations in coagulation system (Koskela et al., 2017).

Studies II and III were carried out on small and heterogeneous patient cohorts, due to the very rare nature of congenital FXIII deficiency and LPI. The small number of patients studied thus limited the use of statistical methods, and probably certain fine-grained, but significant, differences may have been missed. Studies II and III were observational, and thus no causality could be proven.

6.8 Future Considerations

Inspired by our *in vitro* data, we are currently conducting a new study that investigates the systemic effects of SD plasma in a therapeutic plasma exchange (TPE), during the treatment of the neuroimmunological diseases Guillain-Barre syndrome and Myasthenia Gravis. TPE can also be used to treat patients with severe lipemia (Yeh et al., 2003). To date, the ability of TPE to induce systemic changes in

coagulation by modulating the lipid profile has not been elucidated, providing still another dimension for our new studies to evaluate next.

By maintaining FXIII levels above 15 %, bouncing FXIII levels and thus thrombin bursts could be avoided. In theory, this fluctuation of FXIII levels would be achieved by a somewhat more frequent administration of FXIII concentrate, such as once every other week. This procedure would be especially beneficial for patients with congenital bleeding disorders and a history of thrombosis (Szántó et al., 2007).

Our other future endeavors concern LPI. The role of TXA should be studied first *in vitro* and then in existing animal models (Sperandeo et al., 2007). We are also keen on examining the possibilities of using positron emission tomography (PET) in LPI. PET could be used to detect metabolic changes in the liver to determine whether hepatic clearance is impaired in LPI. PET offers the means to investigate our hypothesis without invasive procedures, such as biopsies, which can be highly risky for LPI-associated hemostasis defects. Also, the vascular involvement of the LPI pathology is of interest.

7 CONCLUSIONS

I **The impact of SD plasma, congenital FXIII deficiency and LPI on thrombin generation and fibrinolysis:**
During SD treatment, the levels of the single chain protein S were significantly reduced, leading to a loss of both APC-dependent and -independent protein S activity, and contributed to enhanced TG. In addition, the *in vitro* coagulation phenotype of SD plasma was even further altered by increased fibrinolysis, at least partially due to low levels of α2-AP.

The AT I theory has been somewhat ignored lately, but based on our results, TG increases at low but hemostaticly sufficient FXIII levels. Although low levels of FXIII are sufficient to protect patients against bleeds with steady conditions, the fibrin formed under these circumstances is not optimal. Moreover, fibrin impaired with GPRP also resulted in increased TG, indicating that attenuated fibrin polymerization may lead to the loss of fibrin AT I–like activity and enhanced *in vitro* TG.

In LPI, *in vitro* TG was diminished while *in vivo* TG was increased, whereas fibrinolysis was greatly enhanced.

II **Alterations in clot stability assessed with traditional and functional coagulation assays:**
Combining traditional coagulation screening with functional coagulation assays revealed increased fibrinolysis in SD plasma, while augmented fibrinolysis could be eliminated even at trough FXIII levels in congenital FXIII deficiency. However, increased clot strength was discovered as a response to FXIII concentrate administration. In LPI, OD derivatives confirmed fibrinolysis, which was detected with elevated D-dimer levels. These findings add to the benefits of parallel traditional and functional coagulation screening as means of clot stability evaluation.

III **Recognize factors that contribute to abnormal coagulation associated with LPI and design novel approaches of transfusion therapy accordingly:**

LPI patients are prone to bleeds during invasive procedures. The severity of renal insufficiency correlates with poor fibrin formation and increased fibrinolysis. In addition, poor primary hemostasis, with normal VWF activity were discovered.

Perioperative screening for LPI patients should include total cell count, evaluation of primary hemostasis with PFA-100®, fibrinogen, FXIII and D-dimer levels. Platelet levels should be corrected with transfusions, especially if PFA-100® closure times are prolonged. Likewise, fibrinogen and FXIII levels must be adjusted with concentrates, while fibrinogen and D-dimer levels should be followed in order to monitor the extent of ongoing fibrinolysis.

ACKNOWLEDGMENTS

This study was carried out at the Coagulation Disorders Unit, Division of Hematology, Comprehensive Cancer Center, University of Helsinki and Helsinki University Hospital, Biomedicum Helsinki, and HUSLAB Laboratory Services, Helsinki University Hospital in 2013-2017. I want to thank the personnel at the Coagulation Disorder Unit and HUSLAB Laboratory Services for their kind support and guidance.

Financial support received from the Helsinki University Governmental Grant, The Finnish Society of Anaesthesiologists, Finnish Hematology Association, and Blood Disease Research Foundation is gratefully acknowledged.

First and foremost, I am grateful to my supervisor, Professor Riitta Lassila. You pushed me to think on my own, and that ability is truly the greatest gift a scientist can give to a PhD student. Combining science and clinical work under your guidance has been both rewarding and most inspiring.

Docent Kaj Metsärinne and Docent Jaakko Parkkinen, the official reviewers of this dissertation, had a very profound impact, indeed improving this thesis with their expertise and constructive comments. Professor Eeva-Riitta Savolainen and Docent Tomi Niemi were both members of my thesis committee and are warmly thanked.

I thank all my co-authors, especially PhD Herm-Jan Brinkman from Saquin. Your vast knowledge and professionalism has been highly appreciated. Our collaboration has been an invaluable one for me. I also thank MD, PhD Minna Ilmakunnas and Docent Jouni Ahonen, for their always valuable remarks and encouragement. The third study of this dissertation was executed in collaboration with the Department of Pediatrics, Turku University Hospital. Thus, I want to thank the "LPI team": Professor Harri Niinikoski, Docent Kirsti Näntö-Salonen, MD, PhD Laura Tanner, and MD Mari Kärki for their valued input.

It has been a privilege to work with our group. I thank Vuokko Jokela and Nora Mattila for their company and peer support. I am so very grateful to both Annukka Jouppila and Marja Lemponen. Your professionalism, help, support and always welcome sense of humor always kept me going.

I thank all my colleagues at Vaasa Central Hospital. Your professional guidance will never be forgotten. All my dear colleagues at HUS ATeK Meilahti Hospital and Children's Hospital: You have taught me much. I am living my professional dream because of you. During my years of residency, I met and worked with the best crew on Earth. Thank you, Johanna, Arie, Kariantti, Jani, and Otto. It has indeed been my pleasure to work with you. My special gratitude goes to Heikki Valkonen for his illustrations in this PhD thesis.

I also thank my dear friends from high school and beyond. And, finally, but by no means least, I thank my family. I thank my aunt Paula and my brother, Ville, for their daily help with my beloved cats. Life would have been impossible without you. My deepest gratitude goes to my parents, Pirkko and Kyösti. Your work ethic and morals have guided me throughout my life. You have always supported me to succeed in all my academic goals.

Helsinki, September 2018
Hanna Pitkänen

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Helsinki 2018 ISSN 2342-3161 ISBN 978-951-51-4560-4